An imposter in the nest:

Coevolution between the little bronze-cuckoo and its host, the large-billed gerygone

Hee-Jin Noh

A thesis submitted for the degree of Doctor of Philosophy of
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Declaration

I, Hee-Jin Noh, declare that this thesis, submitted to the degree of Doctor of Philosophy of The Australian National University, is my own original work unless otherwise referenced or acknowledged. All chapters are co-authored. This document has not been submitted for consideration at any other academic institution.

Hee-Jin Noh

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Abstract

Interactions between avian brood parasites and their hosts are one of the most suitable model systems for studying coevolution. Brood parasitic cuckoos lay their eggs in the nests of other bird species, called hosts. In most cuckoo species, the cuckoo chick evicts or outcompetes the host's own brood and becomes the sole occupant of the nest. Therefore, parasitism by cuckoos is highly costly to hosts, selecting for the evolution of host defences. In turn, host defences select for counter-adaptations in cuckoos, giving rise to a coevolutionary arms race.

Many hosts have evolved the ability to recognize and reject mimetic cuckoo eggs, yet fail to discriminate cuckoo chicks. Theory predicts that hosts should not evolve to recognize and reject cuckoo chicks via imprinting because of the risk of mistakenly imprinting on a cuckoo chick in their first brood and thereafter always rejecting their own chicks. However, recent studies have found that the large-billed gerygone (*Gerygone magnirostris*), a host of the little bronze-cuckoo (*Chalcites minutillus*), accepts non-mimetic cuckoo eggs and rejects the cuckoo nestlings. This unique coevolutionary pathway between Australian cuckoos and their hosts has generated much recent discussion in the literature about why cuckoo-host interactions evolve along several different coevolutionary trajectories. In order to address this question, it is necessary to explore the arms race at all stages of the breeding cycle, yet few studies of brood parasites have done this.

The general aim of this thesis is to understand how the reciprocal interactions between one of the virulent avian parasites little bronze-cuckoos and large-billed gerygones have evolved across all breeding stages. I begin with a general introduction to coevolution between cuckoos and their hosts, and an introduction to my study system. In the next two chapters, I focus on defensive strategies in a common host of the little bronze-cuckoo, the large-billed gerygone, throughout the breeding cycle. I begin by examining three frontline defences: nest structure, nest placement and mobbing behaviour (Chapter 1). I test whether nest architecture or position influences the rates of parasitism or predation, and whether gerygones have evolved cuckoo-specific mobbing behaviour or vocalizations using model-presentation experiments.

In Chapter 2, I explore defensive behaviour of large-billed gerygones against little bronze-cuckoos at the egg stage. I test the clutch dilution hypothesis by investigating whether accepting parasite eggs increases reproduction in a multiply parasitized host. Moreover, since large-billed gerygones never reject cuckoo eggs, I investigate whether they have any other form of egg rejection, such as nest abandonment, using field-based observation data.

The aim of Chapters 3 and 4 is to determine whether hosts use visual or vocal cues to discriminate little bronze-cuckoo chicks. In Chapter 3, I use experimental manipulations to test whether gerygones recognize and reject little bronze-cuckoo nestlings based on true template-based recognition of visual cues, or whether they use recognition-free contextual cues. In Chapter 4, I evaluate whether cuckoo begging calls provide a potential additional cue for discrimination of cuckoo chicks by large-billed gerygones, and whether little bronze-cuckoos mimic the begging calls of their hosts. Using a cross-fostering experiment, I then test whether begging calls are plastic, allowing exploitation of multiple hosts, or fixed, potentially constraining the cuckoo to a specialist strategy.

Thesis Outline

General introduction

Chapter 1. Multiple frontline defences against brood parasitic cuckoos in the large-billed gerygone

This chapter is a manuscript currently under preparation for submission

Chapter 2. Multiple parasitism promotes the acceptance of cuckoo eggs in a host

This chapter is a manuscript currently under preparation for submission

Chapter 3. True recognition of nestlings by hosts selects for mimetic cuckoo chicks

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Chapter 4. Imperfect mimicry of host begging calls by a brood parasitic cuckoo and implications for host specialization

This chapter is a manuscript currently under preparation for submission

Synthesis and Conclusions

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General Introduction

Coevolution is the process of reciprocal evolutionary change that occurs between two or more species as a result of their interactions with one another (Thompson 1994, 2009). Beginning with Darwin, the importance of the interactions between organisms for the evolution of species' morphology was recognized (Darwin 1872, 1862) and it was argued that the evolution of plant defences, followed by counter-adaptations in insects, could drive speciation events or extinction (Ehrlich and Raven 1964). In animals, interactions between avian brood parasites and their hosts are one of the most captivating model systems for studying coevolution (Rothstein 1990). Brood parasitic cuckoos lay their eggs in the nests of other species (Davies 2000). In most cuckoo species, the cuckoo chick evicts or outcompetes the host's own brood and becomes the sole occupant of the nest (Davies 2000, Langmore 2013). Therefore, parasitism by cuckoos is highly costly to hosts, and in turn, host defences select for counter-adaptations in cuckoos, giving rise to a coevolutionary arms race (Payne 1977, Rothstein 1990, Davies 2000).

Host defences can be expressed in all stages of the breeding cycle (Feeney et al. 2014). A host of a brood parasitic cuckoo gains greater reproductive success by preventing a cuckoo from laying in her nest than by removing the parasite egg or chick later. This is because female cuckoos typically remove a single host egg when laying their own egg (Brooker and Brooker 1989, Davies 2000). Thus, if a host can prevent a cuckoo from laying in her nest she can retain her entire clutch, whereas if she removes the parasite egg or chick she bears the cost of a reduced clutch. Some hosts have evolved defences that are deployed prior to egg laying by the parasite ('frontline defences'). Mobbing behaviour near a nest can deter female cuckoos from laying eggs, and this is one of the most well-studied frontline defences (Davies 2000). Many bird species mob threatening animals, but close enemy inspection and attack are costly for hosts because they may be dangerous to the adult hosts themselves (Curio et al. 1978). Therefore, mobbing propensity can vary with respect to the degree of threat, and if hosts exhibit specific defensive behaviour to the cuckoos, this can be strong evidence of frontline defence. A second form of frontline defence is adaptation in the design or position of the nest itself, in the same way that nest characteristics play a role in defence against predators. Some hosts build deceptive or decoy nests that prevent or reduce the likelihood of parasitism (Feeney et al. 2012), or build their nests near the nests of aggressive or predatory species, which may discourage brood parasites from approaching the nest (Higgins 2002). In these cases, however, while frontline defences are the most efficient strategy against parasitism, and a role for nest type in reducing parasitism seems intuitive, they

have not been studied in a diversity of species, and few studies have tested this possibility explicitly.

Hosts also express defences at the egg stage of the breeding cycle and the most common of these is egg rejection. Many hosts remove parasitic eggs, or bury them under the nest lining, using cues such as differences in egg colour or size. When egg rejection is not possible, hosts may abandon their parasitized nests (Hosoi and Rothstein 2000, Guigueno and Sealy 2010, Spottiswoode and Stevens 2010). However, some hosts seem to lack the ability to reject foreign eggs even though the cuckoo's eggs may appear very different from their own, and instead they reject cuckoo nestlings (Sato et al. 2010b). The puzzle of why hosts accept cuckoo eggs has been explained by two main hypotheses. First, the evolutionary lag hypothesis states that in some cuckoo-host systems there has not been sufficient time for hosts to evolve discrimination against the parasite (Dawkins and Krebs 1979, Davies 2000). Second, the cost-benefit equilibrium hypothesis states that the host-parasite relationship may evolve toward an evolutionary equilibrium. This is likely to occur when cuckoos have evolved good egg mimicry, leading to a high risk of rejection errors by the host (Davies et al. 1996). In this case the costs of rejection may outweigh the costs of acceptance, so that it is not adaptive for hosts to reject parasite eggs (Davies et al. 1996, Lotem and Nakamura 1998, Takasu 1998), and three possible costs have been proposed: recognition errors, recognition failure and own egg loss from multiple parasitism. Recent research suggests that whether or not hosts commonly experience multiple parasitism in the nest can have important consequences for the evolution of host defences (Davies et al. 1996, Rivers et al. 2012, Gloag et al. 2014). The clutch dilution (or egg dilution) hypothesis is one example of an equilibrium hypothesis. It proposes that in systems where the cuckoo removes a single host egg prior to laying her own egg, and nests are parasitized by multiple parasites, the host may benefit by accepting cuckoo eggs. The benefits of this strategy are predicted to increase with decreasing host clutch size and increasing rates of multiple parasitism (Sato et al. 2010a).

Hosts also express defences against parasitism at the nestling stage. Rejecting parasite nestlings may be based either directly on chick phenotype (true recognition) or on recognition-free cues. Theory predicts that hosts should not evolve to recognize and reject cuckoo chicks via imprinting because of the risk of mistakenly imprinting on a cuckoo chick in their first brood and thereafter always rejecting their own chicks (Lotem

1993). To date, no studies have demonstrated true recognition of parasite young. However, previous work revealed that the nestlings of three bronze-cuckoo species (*Chalcites* spp.) are near perfect visual mimics of the host chicks they exploit, which provides indirect evidence for true recognition because such mimicry would not otherwise be expected to evolve (Langmore et al. 2011, Sato et al. 2015).

Host defences at each stage of the breeding cycle select for counter-adaptations in the parasite. For example, discrimination of cuckoo eggs and chicks by hosts has selected for highly mimetic eggs (Davies and Brooke 1989a) or chicks (Langmore et al. 2011) and mimetic or manipulative begging calls (Madden and Davies 2006, Langmore et al. 2008) as counter-responses in many brood parasite species. In response to these adaptations, hosts have evolved further defence strategies (Davies and Brooke 1989b). First, hosts can evolve a more refined discrimination ability to facilitate recognition of cuckoo egg or chick morphology (Spottiswoode and Stevens 2011). Second, hosts can shift their own egg or chick phenotype away from that of the parasite and other hosts. This often results in polymorphisms among individuals because frequency-dependent selection favours the rarer form (Spottiswoode and Stevens 2011). In the early stages of host-parasite coevolution, a cuckoo may be able to successfully parasitize a range of host species. However, as host defences become increasingly effective, this selects for increasingly host-specific cuckoo counter-adaptations to combat them (Krüger et al. 2009). Eventually, this may select for divergence of the cuckoo into several host-specific races (gentes) or even distinct species (Gibbs et al. 2000, Fossøy et al. 2011), with each gens or species specialising on one host and evolving mimicry of their respective eggs or chicks (Davies and Brooke 1989b). On the other hand, if high parasitism rates cause the decline of a host population, or if the hosts have sufficiently strong egg or chick rejection abilities, the cuckoo gens or species could be driven to extinction if there are no alternative accepter species to exploit (Davies and Brooke 1989b).

The general aim of this thesis is to understand how the reciprocal interactions between Australian cuckoos and their hosts have evolved. In order to fully understand coevolution between brood parasites and their hosts, it is necessary to explore the arms race at all stages of the breeding cycle, yet few studies of brood parasites have done this. In the first three chapters, I focus on defensive strategies in a common host of the little bronze-cuckoo (*Chalcites minutillus*), the large-billed gerygone (*Gerygone magnirostris*), throughout the breeding cycle. The little-bronze cuckoos are highly virulent cuckoos,

because they evict all host young from the nest. In response to rejection of cuckoo chicks by hosts, they have evolved striking visual mimicry of host young. I begin by examining two frontline defences: nest structure/placement and mobbing (Ch. 1). In particular, I test whether nest architecture or position influences the rates of parasitism or predation included in this chapter is a test of whether gerygones have cuckoo-specific mobbing behaviour and vocalizations using model-presentation experiments (a collaborative study with an Honours student). In the second and third chapters, I explore defensive behaviour of large-billed gerygones against little bronze-cuckoos at the egg and nestling stages. I test the clutch dilution hypothesis by investigating whether accepting parasite eggs indeed increases reproduction in a multiply parasitized host (Ch. 2), and I use experimental manipulations to test whether gerygones recognize and reject little bronze-cuckoo nestlings using true recognition (Ch. 3). The aim of Chapter 4 is to examine coevolution from the perspectives of both the large-billed gerygones and the little bronze-cuckoos; specifically, I evaluate whether cuckoo begging calls provide a potential cue for discrimination by large-billed gerygones, and whether little bronze-cuckoos mimic the begging calls of their hosts. Using a cross-fostering experiment, I then test whether begging calls are plastic, allowing exploitation of multiple hosts, or fixed, potentially constraining the cuckoo to a specialist strategy (Ch. 4). Finally, in my last chapter I provide a synthesis of the aims and main findings of this thesis and discuss potential future research. In combination, these chapters provide a broad investigation of coevolution, adaptation, and counter-adaptation between an Australian bronze-cuckoo and its hosts.

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Chapter 1.

Multiple frontline defences against brood parasitic cuckoos in the large-billed gerygone

Hee-Jin Noh[1], Frances Jacomb[1,2], Ros Gloag[3], Naomi E. Langmore[1]

[1] Research School of Biology, Australian National University, Building 116, Daley Road, Canberra, ACT 0200, Australia

[2] Agriculture and Food, CSIRO Black Mountain, Canberra, ACT 2601, Australia (current address)

[3] School of Life and Environmental Sciences, University of Sydney, Macleay Building A12, Science Road, Sydney, NSW 2006, Australia

Abstract

The co-evolutionary arms races between avian brood cuckoos and their hosts may occur at multiple stages of the nesting cycle, beginning with a host's frontline defences - those employed prior to egg laying by the cuckoo. Indeed, selection for frontline defences in hosts should be stronger than that for post-parasitism defences (e.g. rejection of parasite eggs or chicks), because effective frontline defences preserve the entire host clutch. Furthermore, frontline defences against parasites often serve the dual purpose of also reducing nest predation. We investigated whether parasitism by the little bronze-cuckoo (*Chalcites minutillus*) has selected for frontline defences in large-billed gerygones (*Gerygone magnirostris*), a host that rejects cuckoo chicks. We considered three possible defences: nest positioning, nest architecture and host mobbing. We found that 1) gerygones are significantly more likely to build their nest alongside previously-used nests than expected by chance, but nests clustered with old nests were not any less likely to be parasitized or predated, nor did the experimental addition of decoy nests reduce parasitism or predation rates; 2) nest architecture affected parasitism (but not predation) rates, with larger nests being more likely to suffer parasitism than smaller nests; and 3) gerygones exhibited cuckoo-specific mobbing behavior, and were more likely to mob a cuckoo mount than that of a hawk or a harmless sympatric species (willie wagtail). Our results suggest that cuckoo parasitism has selected for a portfolio of defences against cuckoos in large-billed gerygones, comprising both low-cost, but often ineffective, frontline defences (nest design and mobbing), and a high-cost, but highly effective, chick stage defence (chick rejection).

Keywords

frontline defence, brood parasitism, predation, gerygone, bronze-cuckoo

Introduction

Interspecific brood parasites lay their eggs in the nests of other species (hosts) and abandon their young into the host's care (Davies 2000). Hosts suffer high reproductive costs in parasitized nests, losing their young and wasting time and energy in tending to the parasite (Davies 2000). These costs select for defensive host adaptations, which may in turn select for counter-adaptations in brood parasites in a classic example of an evolutionary arms race (Davies 2000). Defences are typically more beneficial to hosts the earlier in the nesting cycle they are expressed (Feeney et al. 2012). For example, a host of a brood parasitic cuckoo gains greater reproductive success by preventing a cuckoo from laying in her nest than by removing the cuckoo egg from the nest after it has been laid. This is because female cuckoos typically remove host egg(s) at the time that they lay their own (Brooker and Brooker 1989, Davies 2000). Furthermore, defences against parasitism that precede the parasite's egg-laying (frontline defences; Feeney et al. 2012) are likely to be common because they often overlap with defences against nest predation, another major cause of reproductive failure (Lima and Dill 1990).

Hosts can reduce the probability of parasitism by physically obstructing a parasite's access to the nest, or by concealing the nest from detection by the parasite (Crook 1963, CoUias and Collias 1984, Davies 2000). In both cases, the design or position of the nest itself may, therefore, be an important form of frontline defence for some hosts. For example, cavity-nesting redstarts (*Phoenicurus phoenicurus*) enjoy low incidences of parasitism by common cuckoos (*Cuculus canorus*) because the cavity entrance is often too small to allow cuckoos access to lay eggs (Rutila et al. 2002). Similarly, there is some evidence that the tube-shaped nest entrance of weaverbirds impedes access by cuckoos (Crook 1963, Davies 2000). Some hosts build their nests near the nests of aggressive or predatory species, which may discourage brood parasites from approaching the nest (Higgins 2002). The yellow-rumped thornbill builds a domed nest with a 'false nest' cup affixed to the top that may deceive cuckoos and predators by creating the false impression of an empty nest (Galligan and Kleindorfer 2008). Finally, the pink-legged Gravateiro (*Acrobatarnis fonsecai*) builds a true nest alongside multiple decoy nests, which may disguise the location of the true nest and thereby reduce predation or parasitism (Whitney et al. 1996). Nest design and characteristics may also play a role in defence against predators. For example, it is widely thought that the function of enclosed nests is to reduce the risk of predation (Nice 1957, Martin et al. 2017). Moreover, many tropical birds build pendant nests over water that look very similar to flood debris and

this resemblance is presumed to reduce predation by acting as a form of masquerade (Collias and Collias 1984, Bruce 2003, Fitzpatrick et al. 2004, Noske et al. 2013). In these cases, however, while a role for nest type in reducing parasitism and predation seems intuitive, no studies have tested this possibility experimentally.

Many bird species mob threatening animals near their nests, but close enemy inspection and attack of some nest predators are costly for hosts because such predators are also dangerous to the adult hosts (Curio et al. 1978). Physically defending a nest can also drive away female cuckoos intending to lay or damage eggs, and this is one of the most well-studied frontline defences (Neudorf and Sealy 1994, Welbergen and Davies 2009, Krüger 2011, Feeney and Langmore 2013, Gloag et al. 2013). One indication that mobbing of cuckoos is a frontline defence against parasitism, rather than a general nest defence, is when cuckoos and nest predators attract different mobbing responses from hosts, appropriate to the threat level posed to adult hosts, in the same way that mobbing can be modulated in response to the risk level and type of predation (Manser 2009). Many birds mob brood parasites, and some exhibit cuckoo or predator-specific mobbing vocalizations and behavior (Gill and Sealy 2004, Manser 2009, Langmore et al. 2012). In one cooperatively breeding host species, mobbing by larger groups was more intense than mobbing by small groups, and large groups were parasitized less often than small groups, suggesting that mobbing provides an effective defence against parasitism (Feeney et al. 2013). In other studies, however, mobbing of brood parasites has been shown to be largely ineffective in preventing parasitism (Gloag et al. 2013, Soler et al. 2014) though it may reduce the opportunity for female parasites to damage host eggs (Gloag et al. 2013).

Here, we studied the role of nest design, nest placement and mobbing behavior against parasitism and predation in the large-billed gerygone *Gerygone magnirostris*, the main host of the little bronze-cuckoo *Chalcites minutillus* in Cairns, Australia. Large-billed gerygones never remove cuckoo eggs, but may reject cuckoo nestlings shortly after they hatch (69% of cuckoo chicks rejected, (Noh et al. 2018). Little bronze-cuckoos have also evolved striking visual mimicry of gerygone chicks (Langmore et al. 2011) in response to host rejection (Noh et al. 2018), suggesting a long co-evolutionary history for this host-parasite pair. Large-billed gerygones build untidy, domed nests of grass and moss, with a long, ragged tail below the nest chamber and a hood concealing the chamber entrance

(Figure 1a). The nests are usually built overhanging water where they resemble flood debris (McGill 1970, Higgins 1999) (Figure 1b). In addition, however, large-billed gerygones sometimes build their active nest alongside abandoned or previously-used nests (Higgins 2002). A study of gerygones in mangrove habitats found that nest predation was significantly less likely at nests that were built close to old nests than those positioned away from old nests, suggesting that old nests act as decoys or otherwise confuse nest predators to the benefit of gerygones (Noske et al. 2013). In addition to nest placement, other aspects of gerygone nest design might influence parasitism and predation rates by reducing detection rates, including the presence of the entrance hood (which helps conceal movement inside the nest), and the overall size of the nest. Interestingly, the nests of large-billed gerygones are substantially larger than those of sympatric, closely related species (e.g. mangrove gerygone *Gerygone levigaster*), and there is great variation in nest characteristics, such as nest size and height, between sympatric gerygone species that also differ significantly in their incidence of cuckoo parasitism (Mulyani 2004, Noske et al. 2013).

In this study, we first assessed whether large-billed gerygones do preferentially place their nests adjacent to other nest-like structures (flood debris or old nests). We then looked for evidence from natural nests that nest placement affected parasitism or predation rates, and tested experimentally whether adding or removing old nests in close proximity to active nests influenced the probability of parasitism or predation. If the main function of placing nests near old nests or food debris is to reduce parasitism or predation, we predicted that the tendency to be depredated or parasitized would be inversely related to the number of nests/debris in the vicinity. Second, we assess whether features of the nest architecture (nest length, hood length and height above ground) influence the probability of parasitism or predation. If so, this suggests that cuckoo parasitism is a current selection pressure shaping nest design. Third, we used model-presentation experiments to test whether gerygones exhibit different mobbing behavior and alarm vocalizations in response to cuckoos, avian predators (hawks) and other non-threatening avian species. A cuckoo-specific mobbing response would indicate mobbing of cuckoos is a form of frontline defence in this host.

Methods

(a) Study system

We studied a population of large-billed gerygones nesting along fifteen creek lines around Cairns City, Queensland, Australia (16°55′ S, 145°46′ E) (see Table S1 for details of study sites). A mobbing experiment was conducted in spring 2015 and a decoy nest experiment and nest measurement were conducted August-December 2017. In Cairns, the main habitat of the large-billed gerygone is freshwater creeks, but they also build their nests in mangrove areas and dry creeks. Cuckoos lay a single egg per gerygone nest, during or shortly after the hosts' egg-laying period, and usually remove one host egg during the same visit. Multiple parasitism of the same nests by multiple cuckoos is common in this system with around one third of parasitized nests receiving two or more cuckoo eggs (Gloag et al., 2014; Noh et al., 2018). The predation rate of large-billed gerygone nests in Darwin was 56.8% (n = 183, Mulyani 2004); the main nest predators were believed to be butcherbirds, small hawks and snakes.

(b) Nest position: proximity to other nest-like structures

To determine whether large-billed gerygones preferred to build nests beside nest-like structures (either old nests or flood debris), we used line-transect surveys to map the location of active nests and nest-like structures along seven creeks in our study area using a global positioning system (GPS; Garmin GPS map 60 CS). Next, we selected five creeks (sites) containing multiple, contiguous large-billed gerygone territories (n = 24 breeding pairs in total) for calculation of average large-billed gerygone territory size. The average distance between synchronous, active nests of these pairs was 72.4m. Therefore, we calculated large-billed gerygone territory size to be approximately 70m (35m either side of the active nest), and we divided each territory into 14 blocks of 5m. We classified each 5m block as containing either; an active nest only, an active nest with flood debris, an active nest with one or more old nests, flood debris only, old nest/s only, or no nest-like structures. For each territory (n = 15), we then conducted two analyses in which we calculated the binomial probability of finding an active nest (expected number) alongside 1) flood debris or 2) old nests in the same 5m block. We used Fisher's exact test to compare the observed versus predicted numbers of nest-like structures beside active nests in each gerygone territory.

To test whether gerygones can reduce parasitism or predation by building their nests adjacent to old nests, we took two approaches. First, we compared parasitism and predation rates of gerygone nests in two unmanipulated groups: (i) those adjacent (<5m) to 2-3 naturally occurring old nests (*n* = 8), and those with no old nests nearby (*n* = 56). Note that the number of nests in the former category was low because many old nests were collected for the experimental treatment (below). Second, we carried out nest-addition and removal experiments to manipulate the number of decoy nests close to each active nest, and then compared parasitism and predation rates to those of our unmanipulated groups. For experimental group 1 (*n* = 18), we added old nests to make a total of four additional decoy nests within 1 m of each nest. The nests were attached to nearby branches with string, and the distances (~1 m) and height differences (~1 m) among the nests simulated those that occur naturally (Figure 1c). For experimental group 2 (*n* = 11) we removed all old nests from within 5m of the active nest prior to the first egg being laid. We monitored the parasitism and predation intensity of all active nests over a two-week period beginning from the laying of the first gerygone egg. Any nests that were flooded or abandoned prior to incubation (for parasitism rates) or prior to day 13 of the nestling period (for predation rates), were excluded from analyses.

(c) Nest architecture

We measured three variables of gerygone nests (*n* = 78) that we hypothesized might affect the visibility and accessibility of the nest to cuckoos and/or predators: total nest length (cm), entrance hood length (cm) and the height of the nest entrance above water or ground (cm). We also classified habitat type and habitat size for each of our 15 sites, because predator species, the density of cuckoos/predators and the visibility of nests are likely to differ according to the habitat type and habitat size. Each site was categorized into one of three habitat types; freshwater creek, dry creek, or mangrove (freshwater creeks = creeks with fresh water, dry creek = no water, mangrove = mangrove trees with salt water), and habitat size was calculated by Google earth v. 7.3. 78 nests were measured and of these, 56 nests were used for the nest addition/removal experiment. We monitored all nests throughout laying and incubation by checking nest contents every 2-3 days and recorded whether or not they were parasitized and/or depredated.

(d) Host mobbing of model cuckoos

In order to investigate whether large-billed gerygones exhibit cuckoo-specific mobbing behaviour, we presented 19 gerygone pairs with mounted, freeze-dried specimens of three bird species. The mounts used were a brood parasite (shining bronze-cuckoo *C. lucidus*, *n* = 17), a predator of both nests and adult gerygones (brown goshawk *Accipiter fasciatus*, or collared sparrowhawk *A. cirrocephalus*, *n* = 19), and a non-threatening control species of a similar size to the cuckoo (willie wagtail *Rhipidura leucophrys*, *n* = 18) (Figure 1).

To control for any possible effect of a particular mount, we used two mounted specimens in each category. We used shining bronze-cuckoo mounts because we were unable to obtain specimens of little bronze-cuckoos, but these species are morphologically very similar and previous studies have shown that shining bronze-cuckoos trigger the same response as the primary bronze-cuckoo parasites in other host species (Payne et al. 1985, Langmore et al. 2012). Likewise, we used each specimen of two morphologically similar hawk species (brown goshawk and collared sparrowhawk), because only a single specimen was available, but both are common predators of passerines in the Cairns region (Aumann 1988, Debus 2012). Willie wagtails are a species of fantail (*Rhipiduridae*) commonly found in the same areas as large-billed gerygones (Higgins 2002). We presented gerygone pairs with all three mount types, with the order of presentation randomized during laying and early incubation periods (<3 days post-incubation), when pairs are most vulnerable to parasitism by cuckoos. Each gerygone pair was used once; although they were not colour-banded, they maintain exclusive territories along creek lines so their identity could be inferred from their location.

To prevent damage from mobbing by large-billed gerygones, we presented mounted specimens in a fine (1.5 cm) wire mesh cage (50 cm (diam.) x 50 cm (ht.)). Prior to trials beginning, we placed the empty cage approximately 2 metres from the nest and left it for 30 minutes to allow the gerygones to habituate to its presence before we put the mount into the cage. We began the trials when gerygones came within 2 m of the mount, a distance within which it could be reasonably assumed that they had seen it. For the next five minutes, we video-recorded behaviour using a Panasonic HC-VX870M video camera positioned at least 10 m from the cage in a hide, or obscured by dense

vegetation. At the same time, we recorded gerygone vocalizations for the duration of the trial using a Sennheiser ME66 microphone and a Marantz PMD660 recorder. Trials lasted 5 minutes, after which we removed the mount. We then waited for one hour before presenting the next mount to reduce the risk of carry-over effects from the previous trial.

From the video recordings, we measured the time spent within 0.5 m of the mount (seconds), which was indicative of mobbing (as all approaches this close to the cage appeared to be mobbing attempts), and the number of vocalizations produced. We identified four distinct types of vocalization based on sonograms (Raven Pro v.1.3, Cornell Laboratory of Ornithology); mobbing call, chit call, squeak call, and song (Figure S1). Mobbing calls were harsh, loud bursts of sound that accompanied swooping or attacking of the cage. Chit calls were short clicking sounds, and were often interspersed with mobbing calls. Squeak calls were short, high-pitched sounds. Song was a melodious warbling of ascending and descending notes. We counted the total number of each call type produced a 5-minute trial and calculated the average call rate per second per gerygone, taking into account whether one or two gerygones were present in the video recording of the trial.

Statistical analyses

(a) Nest position: proximity to other nest-like structures

To assess whether our experimental manipulation of the number of decoy nests influenced the probability of parasitism or predation, we performed binomial generalized linear mixed models (GLMM) using a lme4 package (Bates et al. 2012). As a fixed effect, we entered the treatment (nest addition, nest removal, and two control groups; “control 1”: unmanipulated nests with two or three old nests within 5m, and “control 2”: unmanipulated nests with no old nests within 5m), and included site as a random effect.

(b) Nest architecture

To assess which independent variable best explains our response variable, we derived model-averaged parameter coefficients and standard errors for each predictor variable using cumulative AICc (corrected Akaike information criterion) weights. We kept the five best models and calculated the AICc weight for each variable in the best models to identify the most determinant variables by summing the weight the variable had on these models separately (Table 1). The results did not differ depending on the approach used; the significant effects as identified by the backward-elimination procedure were the same as the results using cumulative AICc. The *dredge* function of the MuMIN package (Barton 2013) were used in R (version 3.4.3).

(c) Host mobbing of model cuckoos

To test whether gerygone movements and vocalizations could be explained by mount type, we followed the procedure of Langmore et al. (2012) and used time spent within 0.5 m of the mount (s) and vocalization rate (calls/s) as response variables. Gamma distribution with a log link and a poisson model were used for analysing time spent and call rate respectively. We included treatment (cuckoo, hawk or wagtail), breeding stage (laying or incubation), and trial order (specimen presented 1st, 2nd or 3rd) as factors in the models. A nest identifier was included as a random term because three trials were conducted at each nest. There were no significant differences in the responses of gerygones to the two specimens in each treatment category (time spent within 0.5m of the mount (s) and vocalization rate, all $p > 0.05$). All statistical analyses were performed using RStudio, software (version 1.1.463).

Results

(a) Does the presence of decoy nests or flood debris near active nests reduce the incidence of parasitism or predation?

Of 96 active nests, we found that 25 (26%) were positioned within 5m of an old nest. Large-billed gerygones built their nests in close proximity to old nests significantly more often than expected by chance (Welch two sample *t*-tests, $p < 0.001$, figure 2a, $n = 78$

nests). However, they did not build their nests close to flood debris any more often than would be predicted by chance (Welch two sample *t*-tests, $p = 0.15$, figure 2b).

We found no difference in the rate of parasitism by cuckoos at gerygone nests at which we had placed additional old nests, nests at which we had removed all nearby old nests, and control nests where neither addition nor removal of old nests occurred (GLMM: $\chi^2_3 = 1.5863$, $p = 0.66$, figure 3a). There were also no significant differences in predation rates among the gerygone nests in our treatments (GLMM: $\chi^2_3 = 1.9954$, $p = 0.57$, figure 3b).

(b) Does nest architecture influence the probability of parasitism or predation?

Gerygone nests showed considerable natural variation in each of the three characteristics that we measured (nest total length - range: 43 - 147cm, SD: 22.87 nest height - range: 109 - 463cm, SD: 22.87, and hood length - range: 14.5 - 107.7mm, SD: 16.71; $n = 78$). Only nest length was a significant predictor of parasitism; longer nests were more likely to be parasitized than shorter nests (parasitized: mean ± s.e. = 90.62 ± 3.45, $n = 57$, unparasitized: mean ± s.e. = 79.45 ± 3.72, $n = 21$, Welch two sample *t*-tests, $p = 0.04$). We failed to detect an effect of hood length, nest height or placement above water versus dry ground on parasitism incidence (Table 1 and 2). Nor were any of the nest characteristics we measured significantly related to the probability of predation (average predation rate prior to 13 days old chicks: 59%). However, habitat type was a significant predictor of predation; nests in freshwater creeks and mangrove areas were more likely to be depredated than nests in dry creeks (Table 1 and 2).

(c) Do gerygones recognize cuckoos as nest threats and do they exhibit cuckoo-specific mobbing behaviors?

Large-billed gerygones mobbed cuckoo mounts more intensively than they did mounts of either a predator (a hawk) or a non-threatening sympatric species (a wagtail) (time spent within 0.5 m of the mount, $F_{(2, 45)} = 22.48$, $p < 0.001$, Figure 4). An approach by a gerygone to within 0.5 m from the mount typically entailed clawing and flapping at the outside of the cage and vocalizing loudly. They also produced significantly more mobbing calls ($F_{(2, 48)} = 67.93$, $p < 0.001$, Figure 5a) and chit calls ($F_{(2, 48)} = 23.90$, $p < 0.001$, Figure

5b) in response to cuckoo mounts than in response to hawk or wagtail mounts. In contrast, gerygones produced significantly more song in the presence of the hawk mount than the cuckoo or control mounts ($F_{(2, 48)}$ = 9.60, p < 0.001, Figure 5c). Squeak calls were produced in response to all specimens, and the rate of these calls did not differ between the three mount treatments ($F_{(2, 48)}$ = 1.16, P = 0.38, Figure 5d).

Neither the amount of time spent at the mount, nor total vocalization rate was affected by breeding stage. The rates of mobbing call, chit call, and squeak call significantly differed with the order of mount presentation; the cuckoo elicited the strongest response regardless of order of presentation for both alarm calls and chit calls, whereas for squeak calls the wagtail elicited the strongest response when it was presented either first or last (mobbing call: $F_{(2, 48)}$ = 26.33, p < 0.001; chit call: $F_{(2, 48)}$ = 19.35, p < 0.001; squeak call: $F_{(2, 48)}$ = 18.70, p < 0.001).

Figure 1. (a) A large-billed gerygone nest, (b) flood debris, (c) an example of the “nest addition” treatment for our experimental test of the effect of decoy nests on parasitism and predation rates of large-billed gerygones (yellow circles: decoy nests and red circle: active nest), and freeze-dried specimens used for mount presentation experiments: d) bronze-cuckoo, e) hawk, and f) willie wagtail

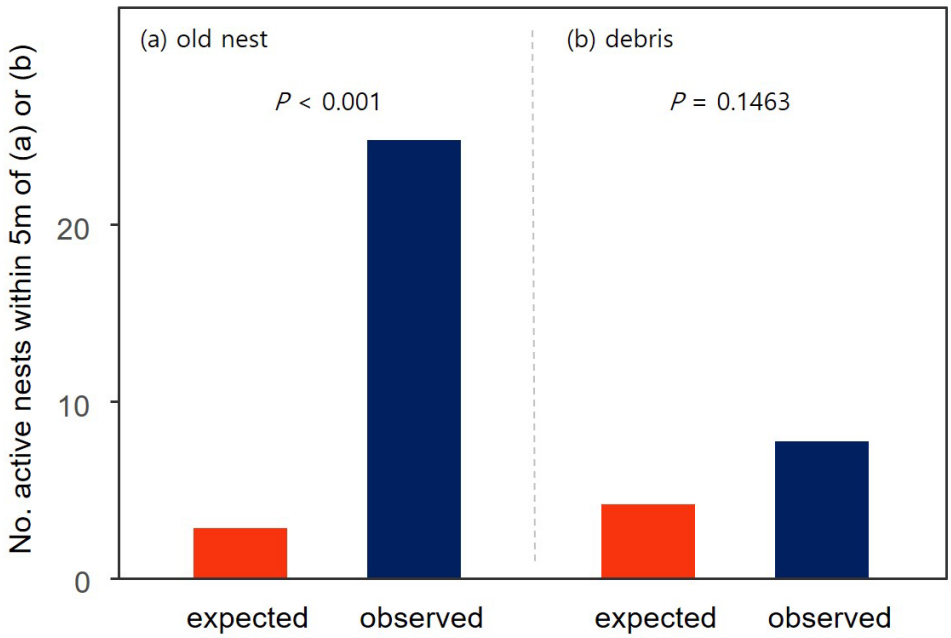

Figure 2. The observed number of active large-billed gerygone nests within 5m of one or more (a) old nests and (b) flood debris at seven creeks in Cairns, and the expected number of nests meeting this condition by chance if nests were placed at random within a gerygone's territory.

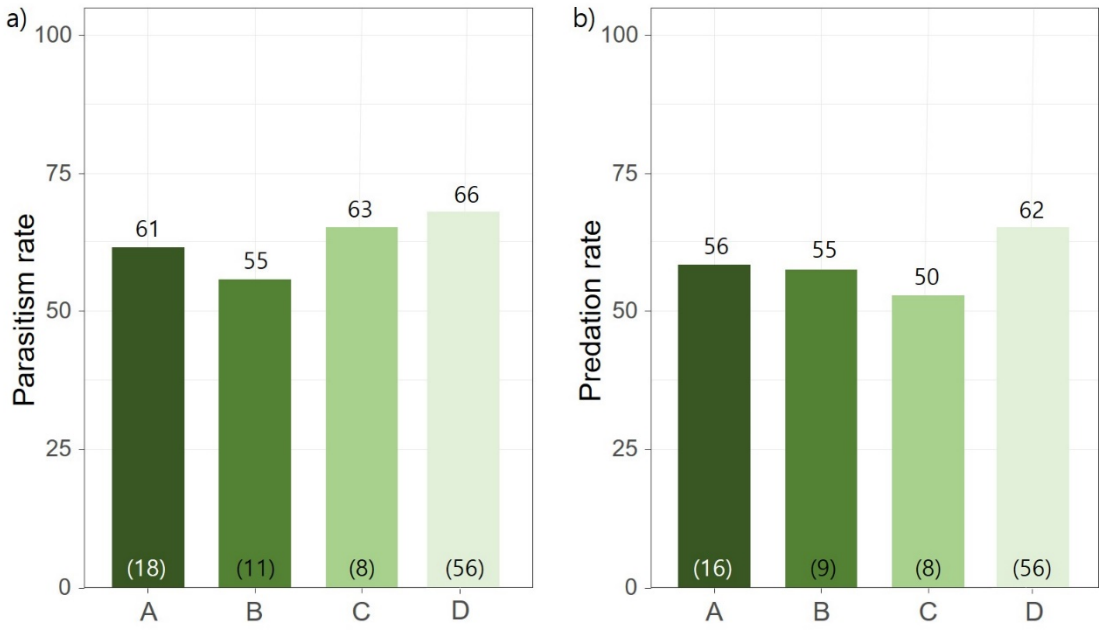

Figure 3. The rate of (a) cuckoo parasitism and (b) predation of nests in four treatments: A: decoy nests added, B: old nests removed, C: control 1 (no manipulation and decoy nests naturally-present), and D: control 2 (no manipulation and decoy nests naturally absent).

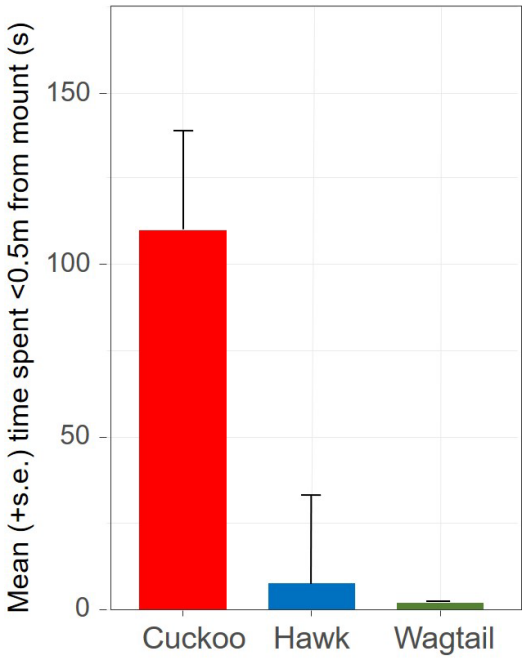

Figure 4. Mean (+ s.e) time (seconds) spent by large-billed gerygones within 0.5 metres of each specimen type (a cuckoo, a hawk and a wagtail) during 5 minutes of mount presentation

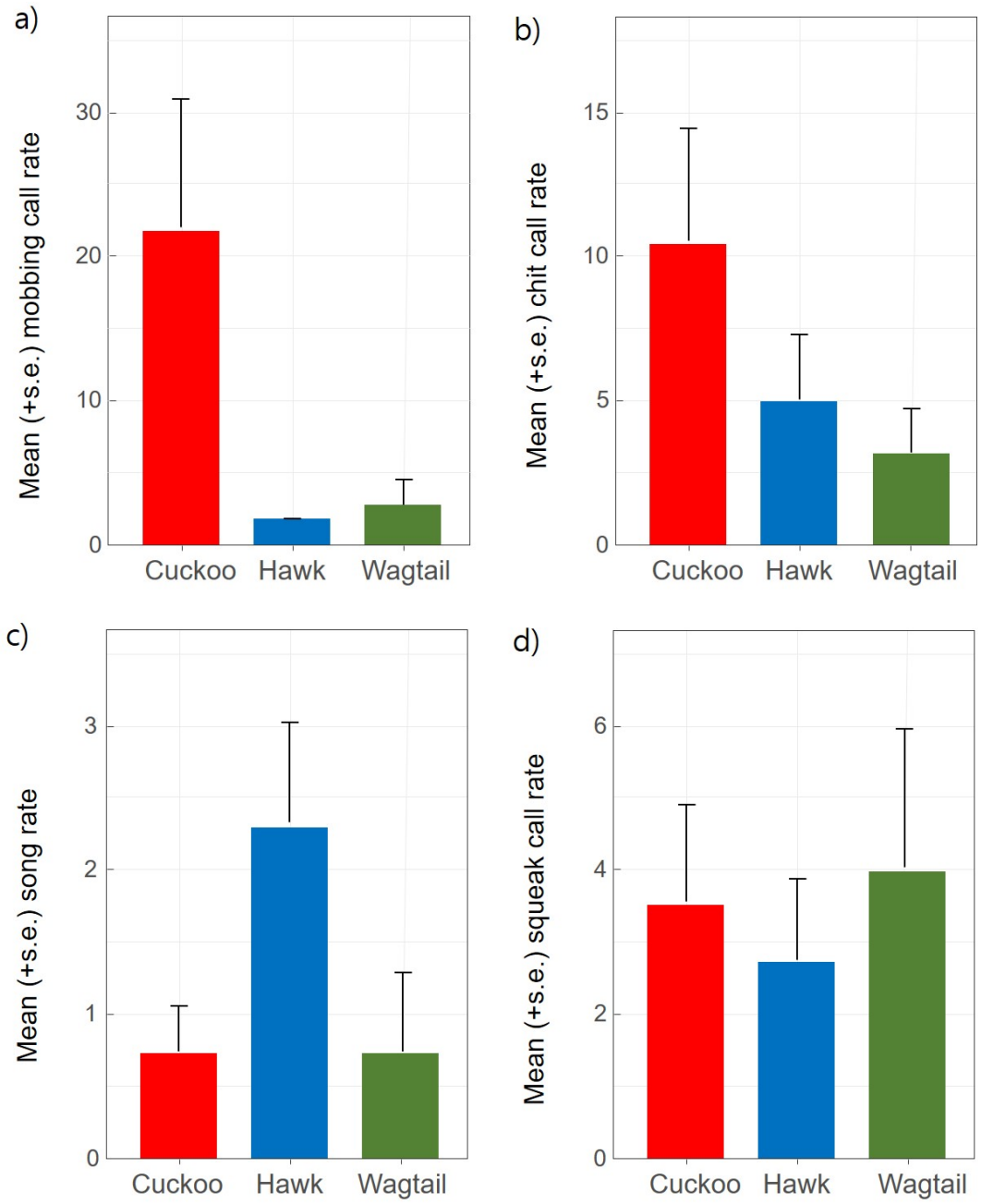

Figure 5. Mean (+s.e.) vocalization rate for a) mobbing calls, b) song, c) chit calls, and d) squeak calls during 5 minutes of presentat ion of three mount types (cuckoo, hawk and wagtail)

Table 1. Candidate models of gerygone nest characteristics for explaining the incidence of parasitism and predation.

	Model	df	logLik	AICc	ΔAIC	AIC weight
Parasitism	habitat types + total nest length	4	-39.86	88.27	0.00	0.34
	total nest length	2	-42.17	88.50	0.23	0.30
	height + total nest length	3	-41.90	90.11	1.84	0.13
	habitat types + total nest length + whether or not the nest was over water	5	-39.74	90.31	2.04	0.12
	total nest length + whether or not the nest was over water	3	-42.10	90.52	2.24	0.11
predation	habitat types	3	-42.87	92.11	0.00	0.39
	habitat types + whether or not the nest was over water	4	-42.40	93.41	1.31	0.20
	habitat types + total nest length	4	-42.66	93.92	1.82	0.16
	height + habitat types	4	-42.86	94.32	2.21	0.13
	habitat types + hood length	4	-42.87	94.34	2.24	0.13

Table 2. The effect of nest characteristics and nesting site habitat of large-billed gerygones on parasitism by little bronze-cuckoos.

				95% CI		
		Estimate	**s.e.**	**LCI**	**HCI**	***p*-value**
Parasitism	habitat types (dry creek)	0	-	-	-	-
	habitat types (freshwater)	-1.13	0.85	-2.83	0.56	0.19
	habitat types (mangrove)	0.45	1.15	-1.85	2.76	0.69
	over water (ground)	0	-	-	-	-
	over water (water)	-0.33	0.76	-1.83	1.17	0.67
	total nest length	0.031	0.015	0.001	0.06	**0.04**
	height	-0.002	0.003	-0.008	0.003	0.47
	hood length	-	-	-	-	-
Predation	habitat types (dry creek)	0	-	-	-	-
	habitat types (freshwater)	2.30	1.09	0.13	4.46	**0.04**
	habitat types (mangrove)	1.73	1.22	-0.70	4.17	0.16
	over water (ground)	0	-	-	-	-
	over water (water)	0.70	0.74	-0.78	2/19	0.35
	total nest length	-1.0008	0.012	-0.03	0.02	0.52
	height	-0.0006	0.003	-0.01	0.01	0.85
	hood length	0.0016	0.016	-0.03	0.04	0.92

Discussion

In this study, we looked for evidence of three types of frontline defences of large-billed gerygones against parasitism: the positioning of nests alongside old nests or flood debris (which may confuse cuckoos searching for nests to parasitize), the architecture of the nest itself (which may conceal the nest or make it difficult to enter) and mobbing by hosts (which may prevent cuckoos from laying eggs or removing host eggs). We first investigated whether the apparent tendency of gerygones to place active nests in the proximity of old nests or flood debris can be considered a form of defence (Noske et al. 2013). We found that 26% of active gerygone nests were clustered together with old nests, and that gerygones built their nests adjacent to old nests, but not flood debris, more often than expected by chance. However, we failed to detect an effect of nest clustering on parasitism or predation rates in unmanipulated nests, nor did our manipulation of the number of old nests near to active nests detect an effect of this nesting habit on the probability of either cuckoo parasitism or nest (egg or chick) predation. Why then do large-billed gerygones build their nests close to old nests? One possible explanation for clustering of nests is that building new nests close to old nests might have an anti-predator function for incubating female gerygones, which are susceptible to predation as their enclosed nests offer little chance of escape if the predator is able to reach the entrance (Noske et al. 2013). Predators landing on, or climbing towards, old nests might cause sufficient movement of the supporting branch to warn the incubating bird in the active nest of impending danger (Noske et al. 2013). We did not see sufficient mortality of adult females in our study to test this possibility. Alternatively, perhaps nests are clustered simply because they are in the most favourable location for nest-building for other reasons, such as proximity to nest-building material or food.

We next looked for evidence that features of the nest design itself influenced parasitism, by associating parasitism rates with the variation in gerygone nests in entrance hood length, nest height above ground and nest size (length). Only the latter trait (nest length) was positively related to the probability of parasitism; longer nests were significantly more likely to be parasitized than shorter nests. A plausible explanation for this result is that larger nests may simply be more conspicuous. Interestingly, however, even though the search patterns of some visually-orientated avian predators should be similar to cuckoos (Söderström et al. 1998), nest size did not detectably affect predation rates in our study. The risk of detection by cuckoos for large nests may instead therefore be

linked to the time taken to build a larger nest. How little bronze-cuckoos find the nests of their hosts and choose suitable nests for parasitism is not known, but available evidence suggests that most cuckoos find host nests by monitoring hosts' activities (Davies 2000, Yang et al. 2017). Therefore, if it takes longer to build a larger nest, the risk that a host will be detected by a cuckoo during nest building will also increase. Given that our intraspecific study found that larger nests were more likely to be exploited by brood parasites, it is unclear why large-billed gerygones, as a species, build a nest that is so much larger than that of other gerygone species. Further research is needed to understand the evolution of the nest architecture in the gerygone clade. Furthermore, it is worth noting that because our assessment of nest traits in relation to parasitism risk only used the existing within-species variation in nests, its power to detect nest features relevant to cuckoo parasitism is limited. This is because critical nest features affecting parasitism (or predation) risk are likely to already be at fixation in the population, provided they are relatively low-cost to construct. For example, although we failed to find an effect of the length of entrance hoods on parasitism or predation rates in our study, the fact that every large-billed gerygone nest has an entrance hood suggests that the hoods themselves (irrespective of length) serve some role in protecting nests or adults. Teasing apart further the extent to which these characters might affect cuckoos in particular would require manipulative experiments (e.g. the experimental removal of hoods). Moreover, there are presumably many trade-offs for gerygones when choosing nesting sites and nest habits, including differences in predator composition between different habitat types.

Finally, we showed that large-billed gerygones mobbed mounts of cuckoos much more than they did the mounts of a dangerous predator (hawk), and a harmless species (wagtail). Gerygones also showed different behaviors and vocalizations in response to the three types of mounts. These results indicate that large-billed gerygones certainly have the ability to categorise threats to the nest into different types and respond accordingly. Interestingly, individual gerygones differed in their tendency to mob; many host pairs exhibited strong mobbing behavior towards cuckoo mounts, while the behavior was entirely lacking in others. Previous studies on fairy-wrens, the host of another bronze-cuckoo, show that recognition of adult cuckoos is learned through individual exposure to cuckoos and observing conspecifics mobbing cuckoos (Langmore et al. 2012, Feeney and Langmore 2013). Even so, once gerygone pairs have learnt to mob cuckoos, the effectiveness of this defence is unclear.

Studies from a range of brood parasitic avian systems suggest that parasitic females are able to sustain high levels of mobbing and still lay their eggs (Welbergen and Davies 2009, Gloag et al. 2013, Gloag et al. 2014), and the handful of direct observations of mobbing by large-billed gerygones suggest this is also true for little bronze-cuckoos (Gloag et al. 2014). However, hosts that observed adult brood parasites had a higher likelihood of successfully rejecting cuckoo nestlings (Noh et al. 2018), and it may be that the main benefit of vigilance prior to egg-laying is to increase the effectiveness of defences that come later in the nesting cycle (strategy facilitation, Kilner & Langmore 2011). Large-billed gerygone defences therefore offer strong support for the idea of strategy facilitation, in which the evolution of one type of host defence favours the evolution of additional defence strategies (Kilner and Langmore 2011, Noh et al. 2018). In this way, our results emphasise the importance of studying adaption and counter-adaptation across all stages of the breeding cycle for understanding the outcomes of coevolutionary arms races between hosts and cuckoos.

Gerygones in our study rarely mobbed the predator (hawk) or control (wagtail) mounts; instead producing significantly more song in response to hawk mounts, and more squeak calls in response to harmless wagtail mounts. Song has been observed in response to predator calls in a number of passerine species (e.g. several fairy-wren species, *Maluridae*, (Langmore and Mulder 1992, Zelano et al. 2001, Greig et al. 2010). No conclusive evidence has been presented for the function of this behaviour, but proposed explanations include pursuit-deterrence (Cresswell 1994), signalling quality to prospective mates (Langmore and Mulder 1992), to summon assistance from conspecifics, or as a non-functional stress response (Mahr et al. 2016). Although gerygones produced some chit and squeak calls in response to wagtails, they otherwise showed little reaction to these mounts, consistent with them regarding wagtails as a low threat to the nest. The functions of chit and squeak calls are not known but they are likely to be part of regular communication between a male and female breeding pair. For example, we observed that chit calls were also produced by adult gerygones when they approached their nests and before/after feeding young chicks.

This study points to recognition and attack of adult cuckoos as a form of anti-parasite defence. Also, we show that there is scope for nest architecture traits to affect parasitism rates. Overall, however, the picture is one of low effectiveness of frontline defences against both parasitism and predation in this host. Gerygones at our study site suffer

high incidences of cuckoo parasitism (~60% of nests), and many parasitized nests receive more than one cuckoo egg (Gloag et al. 2014, Noh et al. 2018). A frontline defence such as mobbing may be relatively "cheap" from the host's point of view, and therefore worthwhile even if it prevents parasitism only rarely or if it facilitates defences later in the nesting cycle.

Ethics

All experiments were conducted under approval of the Australian National University Experimentation Ethics Committee Protocol number A201539 and A2016/16.

Acknowledgements

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Supplementary Material

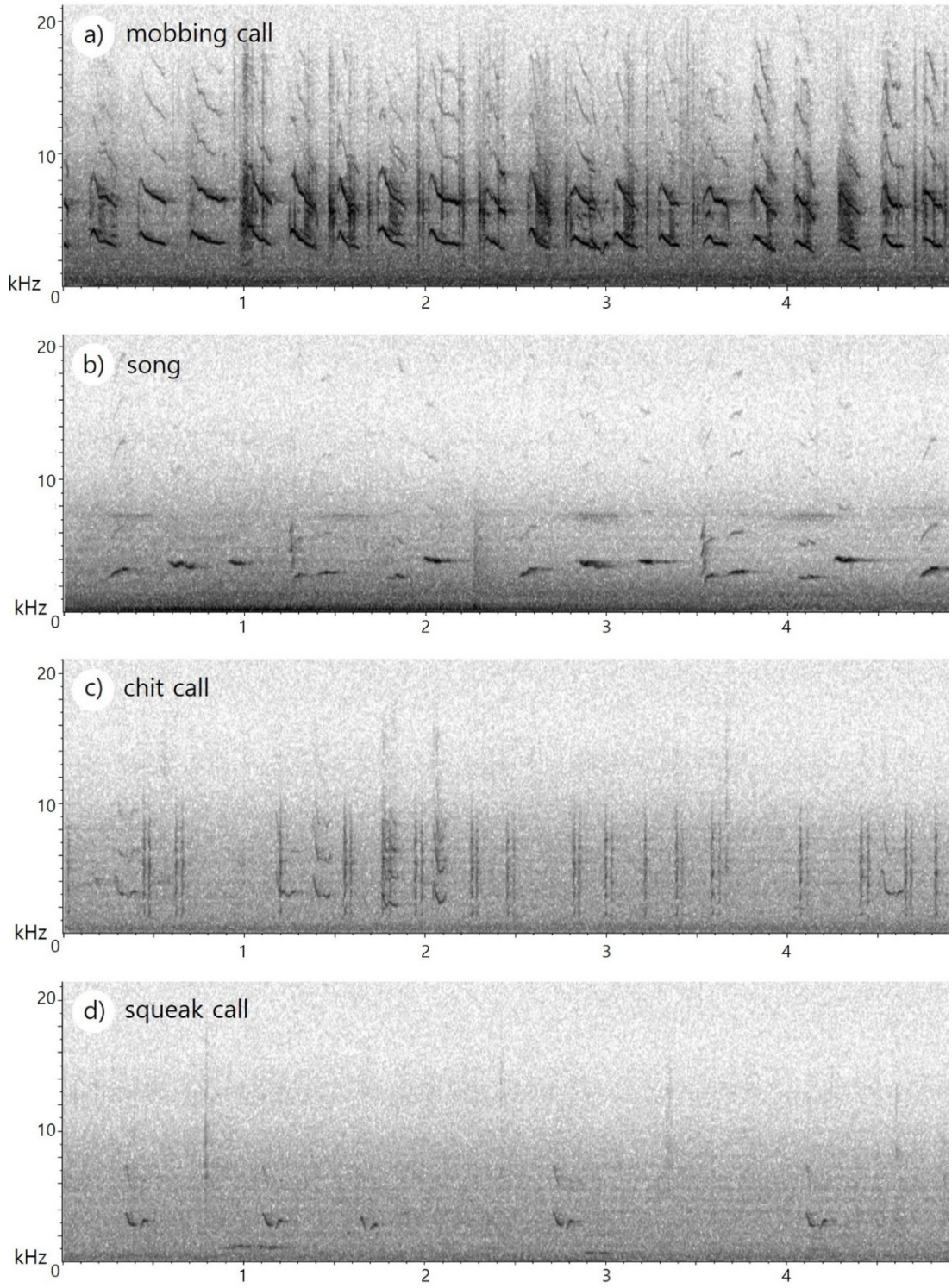

Figure S1. Sonograms of four vocalizations of large-billed gerygone during mount presentation experiments

Table S1. The GPS location, area (m), and habitat types of the study site in Cairns

study area	GPS location	size (m)	habitat type
Crocodile park	16°46'43.63"S 145°40'41.78"E	867	Freshwater creek
Freshwater creek	16°54'20.17"S 145°42'13.01"E	14189	Freshwater creek
Ishmael	16°56'31.31"S 145°44'30.61"E	193	Freshwater creek
Battan park	16°48'11.34"S 145°41'58.42"E	1650	Mangrove
Lily creek	16°54'38.21"S 145°45'29.40"E	617	Mangrove
Botanic garden	16°54'12.95"S 145°45'05.22"E	234	Mangrove
Minnie st	16°55'26.77"S 145°45'37.60"E	690	Mangrove
Trinity beach	16°47'03.45"S 145°41'38.44"E	308	Mangrove
Duune Rd	16°49'40.19"S 145°42'43.75"E	306	Mangrove
Diwi Diwi Rd	16°55'23.34"S 145°44'02.57"E	872	Dry creek
Edmond park	17°00'49.97"S 145°44'02.87"E	2656	Dry creek
Quarts st	17°02'03.45"S 145°44'46.28"E	1214	Dry creek
Horse park	16°55'58.24"S 145°41'24.27"E	256	Dry creek
Cattana wetland	16°49'46.82"S 145°42'11.73"E	57	Dry creek
Carn park	17°01'40.23"S 145°44'38.44"E	2410	Dry creek

Chapter 2.

Multiple parasitism promotes the acceptance of cuckoo eggs in a host

Hee-Jin Noh[1], Ros Gloag[2], Naomi E. Langmore[1]

[1] Research School of Biology, Australian National University, Building 116, Daley Road, Canberra, ACT 0200, Australia

[2] School of Life and Environmental Sciences, University of Sydney, Macleay Building A12, Science Road, Sydney, NSW 2006, Australia

Abstract

Many hosts of brood parasitic cuckoos reject foreign eggs, but the clutch dilution hypothesis posits that where host nests commonly receive more than one parasitic egg, they may benefit by accepting parasite eggs rather than rejecting them. This is because she removes or damages an existing egg when a parasite lays in a host nest. By retaining cuckoo eggs and instead rejecting the cuckoo chick, hosts allow for the chance that later cuckoos remove previously-laid cuckoo eggs, rather than host eggs. We tested this hypothesis in the large-billed gerygone *Gerygone magnirostris*, a host multiply-parasitized by the little bronze-cuckoo *Chalcites minutillus*. Large-billed gerygones do not remove cuckoo eggs from their nests, but instead evict cuckoo chicks. Consistent with beneficial clutch dilution, we find that gerygone egg survival is higher under scenarios of cuckoo egg acceptance than egg ejection. Yet gerygones also showed an adaptive flexibility in their egg acceptance; 35% of gerygones abandoned their nests if they contained only cuckoo egg/s. The absence of host eggs, rather than the presence of cuckoo eggs, appears to trigger nest abandonment. Large-billed gerygones exhibit a sophisticated, facultative response to cuckoo parasitism, switching between cuckoo egg acceptance and rejection to maximize survival of their young.

Keywords

frontline defence, brood parasitism, predation, gerygone, bronze-cuckoo

Introduction

Brood parasitism in birds is costly and often leads to hosts losing all their own brood. Many avian parasites and their hosts are locked in coevolutionary arms races at the egg-stage of the breeding cycle in which hosts are selected to reject foreign-looking eggs from the nest, and parasites are selected to produce eggs that mimic those of the host (Rothstein 1990, Davies 2010, Davies 2011). Yet despite the apparent benefit to hosts of being able to identify and remove parasitic eggs, not all hosts have evolved to reject foreign eggs (Medina and Langmore 2015). One explanation for the lack of this common defence is that, under some conditions, egg rejection itself has significant costs (Davies et al. 1996, Lotem and Nakamura 1998, Takasu 1998). For example, when hosts regularly receive two or more parasite eggs in a nest (multiple parasitism), acceptance of parasite eggs may reduce the risk of host egg loss (Sato et al. 2010a). This is because the parasite typically removes or destroys a single host egg prior to laying her own egg, and so later parasites may remove the eggs laid by earlier ones. Such clutch dilution would be particularly likely to select against the rejection of foreign eggs where host clutch size is small, and rates of multiple parasitism are high (Sato et al. 2010a). Hosts facing these conditions may instead defer rejection of the parasite to the chick stage (Sato et al. 2010a), or invest in other defences that mitigate the costs of rearing parasite chicks (Sato et al. 2010a).

Here, we test the clutch dilution hypothesis in the large-billed gerygone (*Gerygone magnirostris*), the primary host of the little bronze-cuckoo (*Chalcites minutillus*). Little bronze-cuckoos lay a dark olive or brown coloured egg, which is cryptic inside the dome-shaped nests of large billed gerygones and quite distinct from the speckled white eggs of the host (Langmore et al. 2009). Gerygones do not remove these cuckoo eggs (Mulyani 2004, Sato et al. 2010b, Gloag et al. 2014), but it is unclear if this is due to their crypsis, as they also fail to reject experimental eggs painted white (Langmore et al. 2009, Gloag et al. 2014). Clutch dilution offers an additional or alternative explanation for the lack of egg rejection by gerygones, and large-billed gerygones provide a good fit for the conditions of this hypothesis. Although they do not reject cuckoo eggs, large-billed gerygones can rescue a parasitized brood by discriminating cuckoo chicks from host chicks based on morphological differences and then evicting the parasites from the nest soon after hatching (Sato et al. 2010b, Noh et al. 2018). Moreover, large-billed gerygones have a small clutch size of 2-3 eggs and their nests are regularly exploited by multiple female cuckoos (Gloag et al. 2014). Cuckoo egg crypsis may lead second-to-arrive

cuckoos to bias their egg removal toward host eggs, but egg acceptance will still bring a net benefit to gerygones provided cuckoos sometimes remove previously-laid cuckoo eggs (Gloag et al. 2014).

We assessed whether retaining cuckoo eggs in the nest increases gerygone egg survivorship, relative to rejecting cuckoo eggs, by comparing the number of surviving gerygone eggs after second parasitism events in nests. In addition, we extend the clutch dilution hypothesis by considering an additional scenario, in which higher parasitism results in an increased probability of nests containing only cuckoo egg/s. When parasitism rates are consistently high, it is possible that all host eggs are replaced by cuckoo egg/s. In this circumstance, accepting cuckoo eggs would no longer be beneficial, which may drive selection for alternative adaptations in hosts, such as nest abandonment or clutch rejection (both responses to parasitism are observed in other avian brood parasite systems, Langmore et al. 2003, De Mársico et al. 2013). Such a strategy would depend on the host being able to detect that their nest contains either (i) parasite eggs or (ii) no host eggs (the latter being more likely, as cuckoo eggs are cryptic in host nests, Langmore et al. 2009). To distinguish between these two detection cues, we compared responses of hosts to nests containing a) no host eggs and one or more cuckoo eggs, or b) one or more host eggs and one or more cuckoos eggs.

Methods

We conducted fieldwork along creek lines in the Cairns region (16°55′ S, 145°46′ E), Queensland, Australia. We searched for large-billed gerygone nests during the breeding season (Aug-Dec, 2016-2018), and monitored the incidence and intensity of parasitism. Large-billed gerygones lay one egg every second day to produce a typical clutch of 3 eggs (mean: 3±0.09 eggs (range: 1-5, n = 100, (Noh et al. 2018)). During egg-laying, we visited the nests every day and marked eggs to identify them and to check for parasitism, egg rejection, and nest abandonment. We then continued monitoring the nests at intervals of about four- or five-days during the incubation and nestling stages.

To test whether accepting cuckoo eggs decreases the risk of gerygone egg loss, we assessed the number of gerygone eggs remaining in nests after second parasitism for egg accepters and egg rejecters. (1) “Multiple parasitism egg accepters”, (*n* = 13); nests that had already lost one gerygone egg to cuckoo parasitism (i.e. clutches of two gerygone eggs plus a cuckoo egg) at the time of a second parasitism event. After parasitism by the second cuckoo, nests in this group had one of three possible outcomes: (i) two gerygone eggs and a cuckoo egg (the second cuckoo removed the previous cuckoo egg), (ii) two gerygone eggs and two cuckoo eggs (the second cuckoo did not remove an egg), and (iii) a host egg and two cuckoo eggs (the second cuckoo removed one gerygone egg). (2) “Virtual egg rejecters”, (*n* = 14); nests that contained two gerygone eggs at the time of parasitism. This group served to simulate the outcomes for a three-gerygone-egg clutch in which the first cuckoo egg had been rejected rather than accepted (Figure 1-a). In this group there were two possible outcomes: (i) a host egg and a cuckoo egg (the cuckoo removed one host egg), and (ii) two host eggs and a cuckoo egg (the cuckoo did not remove an egg). Cuckoos do not always remove a host egg at the time of parasitism (Gloag, et al. 2014), so there were two possible outcomes after the parasitism event for this group: (i) two gerygone eggs and a cuckoo egg (the cuckoo removed one gerygone egg), and (ii) three gerygone eggs and a cuckoo egg (the cuckoo did not remove a gerygone egg). In a few cases of nests containing 2 gerygone eggs, it was not possible to determine whether a cuckoo had removed a recently-laid 3rd gerygone egg or no egg at all, and these nests were excluded from our analysis *n* = 11). Higher gerygone egg survival in nests of our “multiple parasitism” group than our “virtual egg rejecters” group would support the clutch dilution hypothesis for parasite egg acceptance in this host.

For comparison, we also recorded the number of gerygone eggs remaining in unparasitized nests (“Unparasitized”, *n* = 26; all unparasitized nests with a clutch size of three) and in nests parasitized just once (“Single parasitism”, *n* = 28; nests that contained three gerygone eggs at the time of parasitism by a single cuckoo). Cuckoos do not always remove a host egg at the time of parasitism (Gloag et al. 2014), so there were two possible outcomes after the parasitism event for the “Single parasitism” group: (i) two gerygone eggs and a cuckoo egg (the cuckoo removed one gerygone egg), and (ii) three gerygone eggs and a cuckoo egg (the cuckoo did not remove a gerygone egg). For all groups, the number of surviving eggs was recorded at the end of the first week of incubation. To compare the number of surviving gerygone eggs in our four groups, we used a one-way ANOVA and pairwise comparisons. To test for differences in the

proportion of abandoned nests when there was at least one remaining host egg (a condition possible in both unparasitized and parasitized nests) and when there were only cuckoo egg/s in the nest, we used chi-square tests. To ensure equal opportunity for nest abandonment in all groups, we excluded nests from our dataset that did not survive until at least one week of incubation. All analyses were conducted using R ver. 3.5.3 (R Core Team 2016) and the emmeans packages.

Results

Among all monitored large-billed gerygone nests, 66% (79 of 121) were parasitized by little bronze-cuckoos and 34% (27 of 79) of these were parasitized with two (*n* = 23) or more than two (*n* = 4) cuckoo eggs.

a) Does acceptance of cuckoo eggs increase the average survivorship of gerygone eggs?

The presence of a cuckoo egg in the nest increased the average survivorship of gerygone eggs: multiply parasitized nests retained 1.62 host eggs (± 0.10), and "virtual egg rejecters" (those with only two gerygone eggs at the time of parasitism) retained 1.2 host eggs (± 0.09, *P*=0.013, Figure 1-b). This is because most cuckoos removed an egg at the time of laying (77 of 88 parasitism events) and around half of all second-to-arrive cuckoos removed a previously laid cuckoo egg, rather than a gerygone egg (*n* = 9 of 18 multiple parasitism events). Singly parasitized nests retained 2.18 gerygone eggs (± 0.07, Figure 1-b), and unparasitized nests had close to 100% egg survival (always 3 eggs).

b) Do gerygones eject cuckoo eggs or abandon parasitized nests?

Gerygones never removed host or cuckoo eggs from their nests (unparasitized; *n* = 42, parasitized; *n* = 79). Nor did they ever abandon unparasitized nests containing host eggs (*n* = 42). However, gerygones did abandon some parasitized nests (11%, 9 of 79) and they were far more likely to abandon parasitized nests that contained only cuckoo egg/s

(35%, 8 of 23) than those that retained at least one host egg (1.0%, 1 of 56, χ^2=14.64, df=1, p < 0.000).

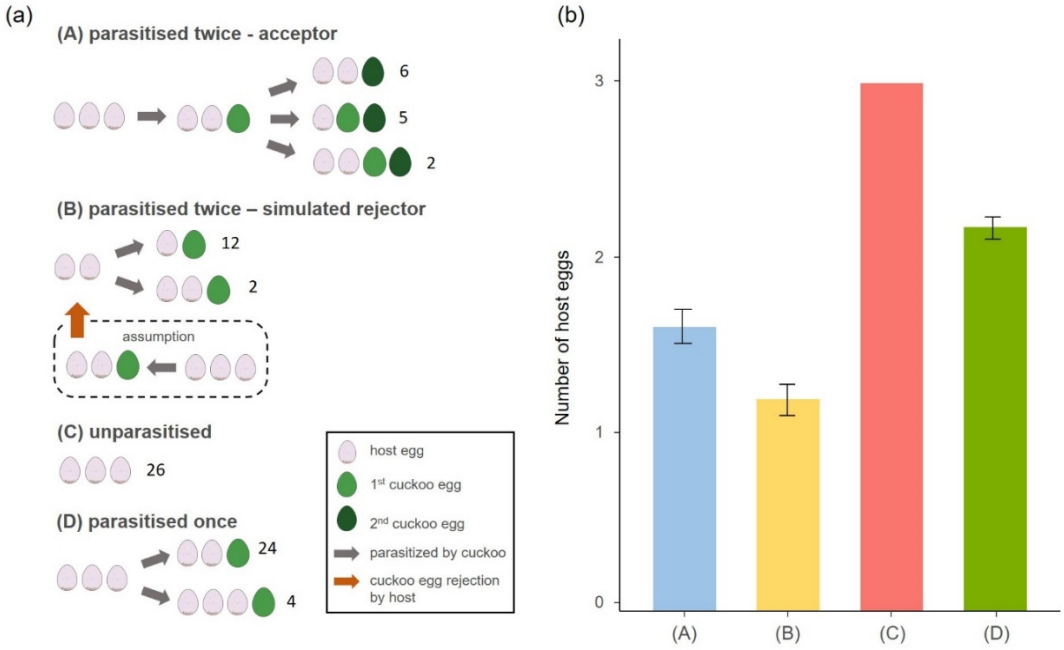

Figure 1. (a) nest compositions of multiple parasitism, and simulated egg rejecter, unparasitized, single parasitism, (b) the number of host eggs left in multiple parasitism (A), and simulated egg rejecter (B), unparasitized (C), single parasitism (D). All groups were significant each other.

Discussion

Egg rejection has evolved repeatedly in many dozens of hosts of avian brood parasites across diverse systems and the egg stage is the stage of the breeding cycle at which the coevolutionary arms race of host and parasite typically plays out. Large-billed gerygones, however, do not eject dissimilar cuckoo eggs (this study, Sato et al. 2010b, Gloag et al. 2014), and instead recognize and remove cuckoo chicks, after which they will proceed to rear any surviving young from parasitized nests (Sato et al. 2010b, Noh et al. 2018). Sato et al. (2010) proposed that multiple parasitism is the key factor driving this unique evolutionary pathway in gerygones, because retaining cuckoo eggs in the nest dilutes the risk that a gerygone egg is removed by another cuckoo. Here we confirm that for multiply parasitized nests of the large-billed gerygone, acceptance of the first-laid cuckoo egg significantly decreases the risk of host egg loss in a subsequent parasitism event, compared to rejection of the first cuckoo egg, consistent with a clutch dilution effect that favours cuckoo egg acceptance.

Does multiple parasitism therefore drive the evolution of chick rejection? Theoretical models propose that only egg acceptors will evolve chick discrimination (Planqué et al. 2002, Grim 2006, Britton et al. 2007). At our study site, a parasitized gerygone nest has a one in three chance of being parasitized again. This high risk of egg loss from second-to-arrive cuckoos is coupled with the high cost of accepting cuckoo nestlings, whose eviction behaviour removes any remaining host brood. In gerygones and other systems with highly virulent parasites, therefore, multiple parasitism may promote chick rejection as the optimal defence strategy. High incidences of multiple parasitism can also favour acceptance of parasite eggs via clutch dilution, however, where parasitic chicks have low virulence. A clutch dilution effect of parasite egg acceptance occurs in mockingbirds parasitized by shiny cowbirds (*Molothrus bonariensis*; Gloag et al. 2011). In this case, protecting their own eggs from repeated female cowbird attacks may ensure that the costs of rearing parasite chicks are kept low, because mockingbird young outcompete cowbird nestmates. Thus, the consequence of multiple parasitism on host defence portfolios varies depending on chick virulence.

In gerygones, the combination of high parasitism rate and small clutch size means that acceptance of cuckoo eggs poses another risk: that the gerygones are left tending a clutch comprising only cuckoo eggs. As might be expected under this maladaptive

scenario, we found that 35% of gerygones deserted their nests in this circumstance. The trigger for nest abandonment appeared to be the absence of host eggs in the nest, because gerygones were significantly more likely to abandon nests containing cuckoo eggs, but no host eggs, than nests containing both cuckoo eggs and host eggs. These results suggest that when parasitism rates are extremely high, it is beneficial for hosts to persevere with any clutches containing at least one host egg, because any new breeding attempt is likely to also be parasitized. Hosts are also selected under such conditions though to recognize and reject nests in which no host eggs remain. Nest abandonment in large-billed gerygones thus appears to be a response to brood parasitism, and shows that large-billed gerygones in fact exhibit plasticity in their egg stage defences, utilizing cuckoo egg acceptance when there is some chance of successfully fledging their own young, and switching to nest abandonment only when there is no possibility of rearing their own chicks.

Ethics

Fieldwork was conducted under approval of the Australian National University Experimentation Ethics Committee Protocol number A201539 and A2016/16.

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Chapter 3.

True recognition of nestlings by hosts selects for mimetic cuckoo chicks

Hee-Jin Noh[1], Ros Gloag[2], Naomi E. Langmore[1]

[1] Research School of Biology, Australian National University, Building 116, Daley Road, Canberra, ACT 0200, Australia

[2] School of Life and Environmental Sciences, University of Sydney, Macleay Building A12, Science Road, Sydney, NSW 2006, Australia

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Abstract

Brood parasitic cuckoos lay their eggs in other birds' nests, whereafter the young cuckoo hatches, ejects its nest-mates and monopolizes the care of the host parents. Theory predicts that hosts should not evolve to recognize and reject cuckoo chicks via imprinting because of the risk of mistakenly imprinting on a cuckoo chick in their first brood and thereafter always rejecting their own chicks. However, recent studies have revealed that some hosts do reject cuckoo chicks from the nest, indicating that these hosts' recognition systems either do not rely on first brood imprinting, or use cues that are independent of chick phenotype. Here, we investigate the proximate mechanisms of chick rejection behaviour in the large-billed gerygone (*Gerygone magnirostris*), a host of the little bronze-cuckoo (*Chalcites minutillus*). We find that gerygones use true template-based recognition based on at least one visual chick trait (the number of hatchling down-feathers), and that this is further mediated by experience of adult cuckoos at the nest during egg-laying. Given the theoretical constraints of acquiring recognition templates via imprinting, gerygones must possess a template of own-chick appearance that is largely innate. This true recognition has facilitated the evolution of very rapid hatchling rejection and, in turn, striking visual mimicry of host young by little bronze-cuckoo chicks.

Keywords

brood parasitism, bronze-cuckoo, gerygone, host defence, chick discrimination, chick rejection

Introduction

Brood parasitic cuckoos impose heavy costs on their hosts, selecting for the evolution of host defences against parasitism (Davies and Brooke 1989a, b, Feeney et al. 2014). The most widespread defence is egg rejection, and many hosts have evolved highly refined abilities to detect and eject eggs that differ in appearance from their own (de la Colina et al. 2012, Hanley et al. 2017). Curiously, however, these same hosts typically fail to reject the parasitic chicks once hatched, despite the imposters having a clearly distinct phenotype from the host's own young (Wyllie 1981, Davies and Brooke 1989b). Several theoretical solutions to this long-standing puzzle have been proposed (reviewed in (Grim 2006)). One explanation is that the costs of recognition errors may constrain the evolution of learned cuckoo chick discrimination in hosts wherever cuckoos evict the host eggs from the nest soon after hatching (Lotem 1993). Lotem suggested that if hosts learn the appearance of their own chicks through imprinting on their first brood, a host parasitized during its first breeding attempt would falsely imprint on the lone foreign chick as its own young and thereafter reject its own offspring for the rest of its life (Lotem 1993). The same problem would not impede the evolution of egg rejection, because even parasitized hosts are exposed to some of their own eggs during the egg-laying and incubation phases.

Lotem's hypothesis provides an explanation for the lack of true learned recognition of cuckoo chicks (assessment of the match between the template for a hosts' own young and the phenotype of the parasite chick) by hosts (Lotem 1993). However, some hosts have evolved the ability to discriminate cuckoo chicks using 'recognition-free' mechanisms (Langmore et al. 2003, Grim 2007, Langmore et al. 2009b). Recognition-free discrimination involves identifying the parasite chick from cues other than chick phenotype, thereby avoiding the risk of mis-imprinting (Grim 2006, Anderson and Hauber 2007, Grim 2007). It has been shown to be the primary process operating in two hosts of evicting cuckoos. Hosts of Horsfield's bronze-cuckoo (*Chalcites basalis*) use the presence of a lone chick in the nest and the presence of adult cuckoos in the population as cues for abandoning parasitized nests (Langmore et al. 2003). Similarly, reed warbler (*Acrocephalus scirpaceus*) hosts of common cuckoos (*Cuculus canorus*) cue into the duration of parental care, abandoning chicks that remain in the nest for longer than the typical host nestling period (Grim 2007). These studies demonstrate that recognition-free discrimination provides hosts with a pathway for cuckoo chick rejection that circumvents the costs of mis-imprinting. Our aim is to test whether cuckoo chick discrimination can

also evolve through true recognition, despite the theoretical costs of mis-imprinting proposed by Lotem (1993). One plausible way in which this could occur is if discrimination is largely innate, rather than learned (Langmore et al. 2009b). In theory, true recognition has a significant advantage over some recognition-free mechanisms, because it can take place immediately upon hatching of the parasite chick, allowing the host to remove the cuckoo before it evicts host young. To date, no studies have demonstrated true recognition of parasite young. However, indirect evidence for this mechanism in some hosts stems from the apparent nestling or fledgling mimicry of host young by cuckoos (Sato et al. 2010b, Tokue and Ueda 2010, Langmore et al. 2011, De Mársico et al. 2012). Just as occurs at the egg stage, selection for mimicry might arise through host rejection of chicks with non-matching phenotypes (Grim 2006, Langmore et al. 2011).

The gerygone (*Gerygone* spp.) hosts of Australia's little bronze-cuckoos (*Chalcites minutillus*) are strong candidates for using true recognition of chicks. Despite typically suffering high parasitism rates, gerygones do not reject bronze-cuckoo eggs (Sato et al. 2010b, Tokue and Ueda 2010, Gloag et al. 2014). Instead, gerygones have the most effective known form of chick rejection because they reject cuckoo chicks by dragging them out of the nest within hours of hatching (Sato et al. 2010b, Tokue and Ueda 2010, Sato et al. 2015), sometimes succeeding in removing the cuckoo nestling (a nest-mate evictor) before it has a chance to evict the host young from the nest. Most Australian bronze-cuckoo species lay non-mimetic eggs, but their chicks are excellent visual mimics of host young, with each subspecies matching the colour of nestling skin, rictal flange and down-feathers of their favoured host species (McGill and Goddard 1979, Langmore et al. 2011, Langmore and Spottiswoode 2012). The little bronze-cuckoo is a particularly accurate mimic of host young (Langmore et al. 2011), and it is unique among cuckoos in displaying multi-barbed nestling down-feathers, which are typical of passerine nestlings including their hosts, but are otherwise unknown in the cuckoo family (Langmore et al. 2011).

Here, we use experimental manipulations to establish for the first time the mechanisms by which gerygones recognize and reject little bronze-cuckoo nestlings. We test for three non-mutually exclusive recognition-free cues (hatch order, the presence of an adult cuckoo and discordancy) and one true recognition cue (nestling down-feathers) that may be used to facilitate chick rejection. The presence of an adult cuckoo in the nest's vicinity

has been shown to be an important component of chick rejection decisions in another bronze-cuckoo host (Langmore et al. 2009b). Hatch order is also a possible recognition-free cue (Shizuka and Lyon 2010), because cuckoo eggs typically require a shorter incubation period and usually hatch 1–2 days before gerygone young (Davies 2011). However, this cue would only be useful in conjunction with another cue, indicating that the nest has been parasitized. Recognition by discordancy involves assessment of the differences between chick phenotypes within the same brood and rejection of the least common phenotype (Rothstein 1974, Moskát et al. 2010). In the absence of true recognition, discordancy might favour visual mimicry of host young by cuckoos, provided that the cuckoo and host chicks are present in the nest together prior to rejection, and that host chicks typically outnumber cuckoos.

Material and methods

(a) Study area and species

We carried out our study from August to December 2016 along creeklines in and around Cairns, Queensland, Australia (16°55′ S, 145°46′ E) on a population of large-billed gerygones (*Gerygone magnirostris*) that experience high rates of parasitism by little bronze-cuckoos (63–65% (Gloag et al. 2014); this study). Little bronze-cuckoos were seen or heard, and parasitism occurred, at all creeks in the study. The large-billed gerygone builds untidy domed nests using grass, moss and spiders' egg-sacks, usually over-hanging water (Higgins 1999). Gerygones lay one egg every second day over a period of 4–8 days (average clutch: mean ± s.e. = 3 ± 0.09, range: 1–5, n = 100) and start incubation when their clutch is complete (Higgins 1999). Cuckoos lay a single egg per host nest, during or shortly after the hosts' egg-laying period, and usually remove one host egg during the same visit. Two or three different females may lay in the same host nest (Langmore et al. 2009a, Gloag et al. 2014). Hosts mob the cuckoo if it is detected during laying, but mobbing has not been observed to prevent parasitism (Gloag et al. 2014).

(b) General experimental methods

We located 54 large-billed gerygone nests during the nest-building phase by searching along creeks, rivers and lakes. Of these nests, 35 (65%) were subsequently parasitized by one (n = 30) or two (n = 5) cuckoos, and 19 were not parasitized. We checked nest contents daily to allow clutch manipulation as soon as eggs appeared and before incubation began. From hatching day, we monitored all 54 nests to determine whether nestling rejection occurred. Parasitized nests were filmed or observed continuously from hatching until host chick eviction (by cuckoo chicks) or cuckoo chick rejection (by host parents) occurred. When cuckoo chicks evicted host young and became the sole occupant of the nest, we continuously monitored the nest for at least a further 2 days during daylight hours to document any chick rejection or nest predation. In total, 19 nests (16 parasitized and three unparasitized) were monitored from 06.00–07.00 to 17.00–18.00 by an observer in a hide (approx. 5 m from nest) using binoculars, 17 nests (16 parasitized and one unparasitized) were filmed continuously with a video camera (Panasonic, HC-VX870M) and the remaining 20 nests (three parasitized and 15 unparasitized) were monitored with daily nest checks to determine whether any chicks were missing. If a host chick was missing from an unparasitized nest, we concluded that the host had rejected the chick. This conclusion is based on the lack of observations of partial predation in our study site (other than when an egg was stuck to or embedded in the lining of a depredated nest) and the fact that nest predation usually results in nest damage as the predator forces entry into the dome nest. If a nest check revealed that all chicks in the nest were missing, we concluded that the nest had been predated. We excluded three unobserved disappearances of cuckoo chicks from our analyses, because in each case the cuckoo chick was alone in the nest so we could not determine whether it disappeared due to ejection or predation. Three host chicks that died in the nest on the day after hatching day were excluded from the analysis because we have no evidence either for or against the idea that dead chicks were rejected. We calculated ‘time to ejection’ to the nearest day (hatch day = 0 days). In our experiments, we first manipulated exposure of hosts to an adult cuckoo using a cross-fostering experiment, and then randomly assigned nests to (i) a hatch-order manipulation experiment, (ii) a feather trimming experiment, or (iii) both the hatch order and the feather trimming experiment (see electronic supplementary material, figure S1).

(c) Manipulation of opportunity to observe an adult cuckoo at the nest

To assess whether hosts' exposure to an adult cuckoo at the nest influenced chick rejection rates, we cross-fostered cuckoo eggs from some parasitized nests to unparasitized nests to create two conditions among nests containing one or two cuckoo eggs: naturally parasitized, such that parents had the opportunity to observe a cuckoo lay at their nest (nests: n = 22, chicks: n = 25), and artificially parasitized nests where adults did not see a cuckoo lay in their nest (nests: n = 13, chicks: n = 15). Two sources of evidence suggest that parents of naturally parasitized nests are likely to have had the opportunity to observe a cuckoo entering the nest. First, we filmed parasitism of the nest on three occasions and, in every case, the gerygone parents mobbed the cuckoo (Gloag et al. 2014). Second, although we cannot be certain that all naturally parasitized hosts observed the cuckoo during parasitism, it is certain that more gerygones in the 'naturally parasitized' group will have seen or interacted with a cuckoo at their nest than did gerygones in the 'artificially parasitized' group.

(d) Manipulation of hatch order and discordancy

Cuckoo eggs usually hatch 1–2 days before host eggs (Davies 2010). To determine the effect of hatch order on chick rejection, we delayed the hatching of cuckoo eggs (n = 12) by 5 days. We removed each freshly laid cuckoo egg and stored it in a cool, dark place. We replaced it temporarily with a non-viable gerygone egg, which had been collected from a depredated or abandoned nest (depredated nests sometimes contained intact eggs, if they were stuck to the nest lining). After 2 days of incubation, we removed the dummy egg and returned the cuckoo egg to the nest, such that any host eggs in the nest hatched 1–2 days before the cuckoo chick. As a control, we used the same procedure to remove and later replace a single gerygone egg from unparasitized nests (n = 14). Five cuckoo chicks also hatched later than host young naturally and these were included in the dataset.

When a cuckoo chick or chicks are the minority species in the brood, hosts may discriminate via discordancy and reject the most dissimilar chick or chicks. This recognition-free mechanism requires that cuckoo and host chicks are present in the nest at the same time, and that host chicks reliably outnumber cuckoos. In combination, our cross-fostering and hatch-order manipulations varied the composition of chicks in the

nest at the same time, and thus allowed us to test for evidence of discordancy as a rejection cue by comparing rejection rates when the cuckoo chick was (n = 8) or was not (n = 32) the brood's minority species.

(e) Manipulation of chick morphology

To assess whether gerygones' rejection of cuckoo chicks is based on true recognition, and specifically on the recognition of nestling down-feathers, we manipulated hatchlings' feathers in a subset of nests (n = 32). On the day of hatching, we used nail scissors to trim the down-feathers of either one cuckoo chick (n = 13, including one naturally naked cuckoo chick) or one gerygone chick (unparasitized nests: n = 16, parasitized nests: n = 4) in the nest (figure 1). We compared the rate of rejection of trimmed chicks with that of chicks that were handled on hatching day, but did not have their feathers trimmed. We also counted the number of down-feathers of all chicks on hatch day, prior to the manipulation, to quantify natural variation in feather density and weighed chicks four times (hatching day, and 3, 7, and 13 days old) to test whether the manipulation otherwise affected chick growth.

(f) Statistical analyses

We used a generalized linear model (GLM) with a binomial distribution and a logit link using all chicks (full dataset) to assess parental responses to the chicks (accept/reject) according to (i) the presence or absence of down-feathers, (ii) hatching order, (iii) whether or not host was exposed to adult cuckoo and/or (iv) whether or not a cuckoo chick was in the minority in the nest. The independent variables were species (cuckoo or host), the four manipulations (all scored as yes/no: hatched first, feathers trimmed, naturally parasitized cuckoo visited the nest and cuckoo chick in the minority), hatching date and the two-way interactions between these variables. We also tested the quadratic term for the hatching date because seasonal trends are often nonlinear, but the result was the same. Initially, we attempted to run a mixed model controlling for nest identity as a random effect because there were multiple chicks in each nest, but this made the model unstable due to the small number of replicates in each nest. Instead, we ran a binomial GLM with a logit link function on a reduced dataset comprising only one experimental chick per nest (reduced dataset) and then compared the results from the full dataset and the reduced dataset. Where there was only one manipulated chick in the

nest, this was included in the reduced dataset. If there were two experimental chicks or there was no manipulated chick in the nests, we selected one chick randomly. In addition, to identify which of these factors contributed significantly to the time to rejection, we used a GLM with a binomial distribution depending on whether or not they were rejected on the day of hatching using all rejected chicks. The independent variables were the same as in the former GLM analysis. Owing to the controversy over whether null hypothesis testing or information theoretic approaches are better for analysis of experimental studies (Murtaugh 2014), we used both methods. We applied a backward-elimination procedure (tables 1 and 2), and the Akaike information criterion (AIC) was also used to support selection of the final model (best-fit model) (electronic supplementary material, tables S1 and S2). The results did not differ depending on the approach used; the significant effects as identified by the backward-elimination procedure were the same as the best model using AIC. We also evaluated multicollinearity using the variance inflation factor (VIF) in the models, and all VIF values were lower than the suggested threshold (greater than 10 (Zuur et al. 2010)). All statistical analyses were performed using R software v. 3.4.3 (R Core Team 2016).

Results

Our full dataset (all chicks in experimental nests) included 85 host chicks and 40 cuckoo chicks across 54 nests. During the course of our experiment, 36 chicks (both host and cuckoo) were rejected from 32 nests (although host chicks were only rejected following down-feather manipulations; see below). We captured nine rejection events on film at eight nests (see example in electronic supplementary material, video S1), and a further five rejection events were observed with binoculars. The remaining chick rejections by hosts were inferred from daily nest checks. In all filmed or observed cases, large-billed gerygones pulled living chicks out of the nests, and the parents then continued to care for the remaining eggs and nestlings. Ejected chicks were either dropped just under the nests or carried up to 3 m from the nest before being dropped.

(a) True recognition

Chick species and the presence of nestling down-feathers were significant predictors of their rejection by gerygone hosts; cuckoos were more likely to be rejected than host

chicks, trimmed chicks more likely to be rejected than untrimmed chicks in cuckoos and untrimmed host chicks were never rejected (table 1 and figure 2a). Hosts removed 93% of trimmed cuckoo chicks (13 of 14 chicks) and 55% of trimmed host chicks (11 of 20 chicks; figure 2a). Among untrimmed chicks, 50% of cuckoo chicks were rejected (14 of 28), while untrimmed host chicks were never removed (n = 65 chicks, figure 2a), and trimming was the only manipulation that resulted in hosts rejecting their own chicks. Where cuckoo chicks did not have their down-feathers manipulated, host parents showed a non-significant tendency to reject those that had naturally fewer down-feathers (rejected: mean ± s.e. = 13 ± 1.77, n = 14; accepted: mean ± s.e. = 17.07 ± 1.67, n = 14; Student's t-test: t = −1.67, p = 0.1068). Similarly, our results from the reduced dataset showed that chick species and the presence of down-feathers were the most significant predictors of rejection (table 1).

The timing of nestling removal further supports a role for direct species-specific chick cues in gerygones' rejection decisions. Cuckoo chicks were more likely to be rejected on the hatch day than host chicks, but whether or not a chick was trimmed did not influence the timing of its removal (table 2). All rejected cuckoo chicks were removed by hosts within 2 days of hatching, with 56% (14 of 25) and 36% (9 of 25) rejected on hatching day and the next day, respectively. Only 8% (2 of 25) were rejected 2 days after hatching (electronic supplementary material, figure S2). By contrast, just 18% (2 of 11) of rejected host chicks were ejected on hatch day, 36% (4 of 11) were removed the day after hatching and 46% (5 of 11) were rejected 2–3 days after hatching (electronic supplementary material, figure S2). In the case of cuckoo chicks, rapid rejection was necessary to preserve the host young in the nest: when hosts removed cuckoo chicks on hatch day, none of their own nestlings had yet been evicted by the cuckoo chick, while those cuckoo chicks rejected on later days had already removed some or all gerygone young (six out of 11).

Finally, we confirmed that hatchling down-feathers vary under natural conditions in both gerygone and cuckoo young. On average, large-billed gerygone nestlings had more down-feathers on the day of hatching (mean ± s.e. = 37.35 ± 1.45, range: 23–68, n = 41, one chick per nest) than little bronze-cuckoo nestlings (mean ± s.e. = 14.40 ± 1.19, range: 0–29, n = 38, Student's t-test: t = −12.226, p < 0.0001). The variation in the number of down-feathers of gerygone chicks within the same brood was significantly less than that between broods (one-way ANOVA: F40,44 = 5.35, p < 0.0001).

(b) Recognition-free cues in host decisions to reject chicks

In naturally parasitized nests in which hatch order was not manipulated (n = 22), the cuckoo hatched before the host chicks in 77% of cases, but based on experimental nests, hatch order had no significant effect on the probability of chick rejection (table 1). Whether the cuckoo chick was in the minority in the brood also did not influence chick rejection decisions (table 1). In addition, hatch order and whether or not a chick is in the minority in the brood did not affect the timing of its removal (table 2).

Host's rejection decisions were influenced in part, however, by the exposure to adult cuckoos in interaction with chick phenotype (table 1), with hosts more likely to reject a cuckoo chick if it had been laid naturally into the nest than if it had been cross-fostered there from another nest by us (table 1 and figure 2b). This was clearly evident among the sample of untrimmed cuckoo chicks; only 18% of untrimmed cuckoo chicks (two out of 11) from artificially parasitized nests were ejected, whereas parents that had the opportunity to observe adult cuckoos laying rejected 69% of untrimmed cuckoo chicks (11 out of 16; Fisher's exact probability test: $p < 0.01$; figure 2b). However, our results from the reduced dataset showed that the effect of whether the nest was parasitized naturally or artificially was trivial (table 1), presumably due to dataset sample size differences.

Table 1. Effects of experimental treatment on chick rejection. Acceptance or rejection of nestlings was modelled as a binomial in a GLM with a logit link function on both the full dataset and the dataset including only one manipulated chick in each nest (results in parentheses). Significant p-values are shown in italic.

term	effect	estimate	s.e	95% CI		deviance	*p-values*
				LCI	UCI		
included	species (cuckoo)	-1.578 (-0.264)	0.777 (0.506)	-3.454 (-1.122)	-0.239 (0.565)	31.667 (5.969)	*<0.0001* (*0.015*)
	species (host)	-6.020 (-3.170)	1.370 (1.080)	-9.520 (-5.498)	-3.861 (-1.702)		
	trim (y)	6.252 (3.174)	1.441 (1.084)	3.893 (1.675)	9.840 (5.500)	41.478 (16.372)	*< 0.0001* (*< 0.0001*)
	exposure to adult cuckoo (y)	2.269 (1.273)	0.924 (0.939)	0.601 (-0.187)	4.355 (2.994)	2.108 (2.037)	0.146 (0.154)
	trim (y) : exposure to adult cuckoo (y)	-4.109 (-3.069)	1.62 (2.267)	-7.798 (-7.131)	-1.1690 (0.581)	3.920 (-1.924)	*0.005* (0.165)
excluded	hatch order (first)	-0.0144 (-0.828)	0.6391 (0.794)	-1.094 (-2.197)	1.027 (0.444)	-0.001 (-1.130)	0.982 (0.288)
	discordancy (y)	0.388 (0.837)	0.764 (0.844)	-0.868 (-0.526)	1.673 (2.287)	-0.259 (-1.011)	0.6111(0.315)
	hatching date	0.013 (0.011)	0.012 (0.014)	-0.006 (-0.013)	0.035 (0.037)	-1.287 (-0.572)	0.256 (0.450)
	species (h) : hatch order (f)	-2.864 (3.100)	3831.454 (1.780)	-80.844 (0.343)	75.116 (6.331)	-0.000 (-3.458)	1 (0.063)
	species (h) : trim (y)	17.168 (34.63)	2045.330 (9300)	-77.750 (-)	515.633 (-)	-1.859 (-0.826)	0.173 (0.364)
	species (h) : exposure to adult cuckoo (y)	-2.332 (16.335)	3488.828 (2655.399)	-67.924 (-)	59.085 (-)	0.000 (-0.676)	1 (0.411)
	species (h): discordancy (y)	-17.307 (-18.476)	2327.470(2711.847)	-634.712 (-)	84.847 (-)	-1.693 (-2.094)	0.193 (0.150)
	trim (y) : hatch order (y)	-1.510 (-19.058)	1.702 (4091.637)	-4.614 (-)	1.166 (-)	-0.839 (-1.690)	0.360 (0.194)
	trim (y) : discordancy (y)	1.659 (-)	17970 (-)	-327.640 (-)	344.594 (-)	-0.000 (-)	1 (-)
	hatch order (y) : exposure to adult cuckoo (y)	-1.326 (14.62)	1.370 (15490)	-3.679 (403.428)	0.886 (360.749)	-0.963 (0.000)	0.326 (1)
	hatch order (y) : discordancy (y)	3.115 (0.967)	1.991 (1.822)	-0.026 (-2.071)	6.700 (4.015)	-2.659 (-0.281)	0.103 (0.596)
	exposure to adult cuckoo (y) : discordancy (y)	-1.258 (11.97)	1.918 (30940)	-4.531 (-804.412)	2.061 (780.835)	-0.422 (-0.000)	0.516 (1)

Table 2. Effects of experimental treatment on time until rejection for those nests in which hosts rejected a chick. Time to rejection was modelled as a binomial (hatching day = 0, one or more days post-hatching day = 1) in a GLM with a logit link function and the dataset includes all the rejected cuckoo and host chicks. Significant p-values are shown in italic.

term	effect	estimate (s.e)	95% CI		deviance	*p-value*
			LCI	UCI		
Included	species (c)	0.241 (0.403)	-0.418	0.9189	45.780	*0.008*
	species (h)	-1.792 (0.764)	-3.2918339	-0.682		
excluded	trim (y)	-0.94225 (0.920)	-2.547	0.528	-1.095	0.295
	hatch order (f)	0.5416 (0.900)	0.282	0.890	-0.368	0.544
	exposure to adult cuckoo (y)	-1.1376 (0.981)	-2.961	0.366	-1.508	0.219
	discordancy (y)	-1.2117 (1.211)	-3.652	0.629	-1.136	0.287
	hatching date	0.010 (0.017)	-0.017	0.039	-0.368	0.544
	species (h) : trim (y)	1.005 (4696)	-	-	-0.000	1
	species (h) : hatch order (y)	1.854	-	-	-2.866	0.091
	species : exposure to adult cuckoo (y)	54.95 (21670)	-	-	0.000	1
	species : discordancy (y)	20.54 (4212)	-	-	-2.885	0.089
	trim (y) : hatch order (y)	18.820 (5628)	-	-	-1.253	0.263
	trim : exposure to adult cuckoo (y)	15.848 (4027.416)	-	-	-0.272	0.602
	hatch order (f) : exposure to adult cuckoo (y)	16.920 (3810.961)	-	-	-0.624	0.429
	hatch order : discordancy (y)	2.117 (24670)	-	-	-1.8052e-09	1
	exposure to adult cuckoo (y) : discordancy (y)	55.100 (32090)	-	-	-1.7227e-08	1

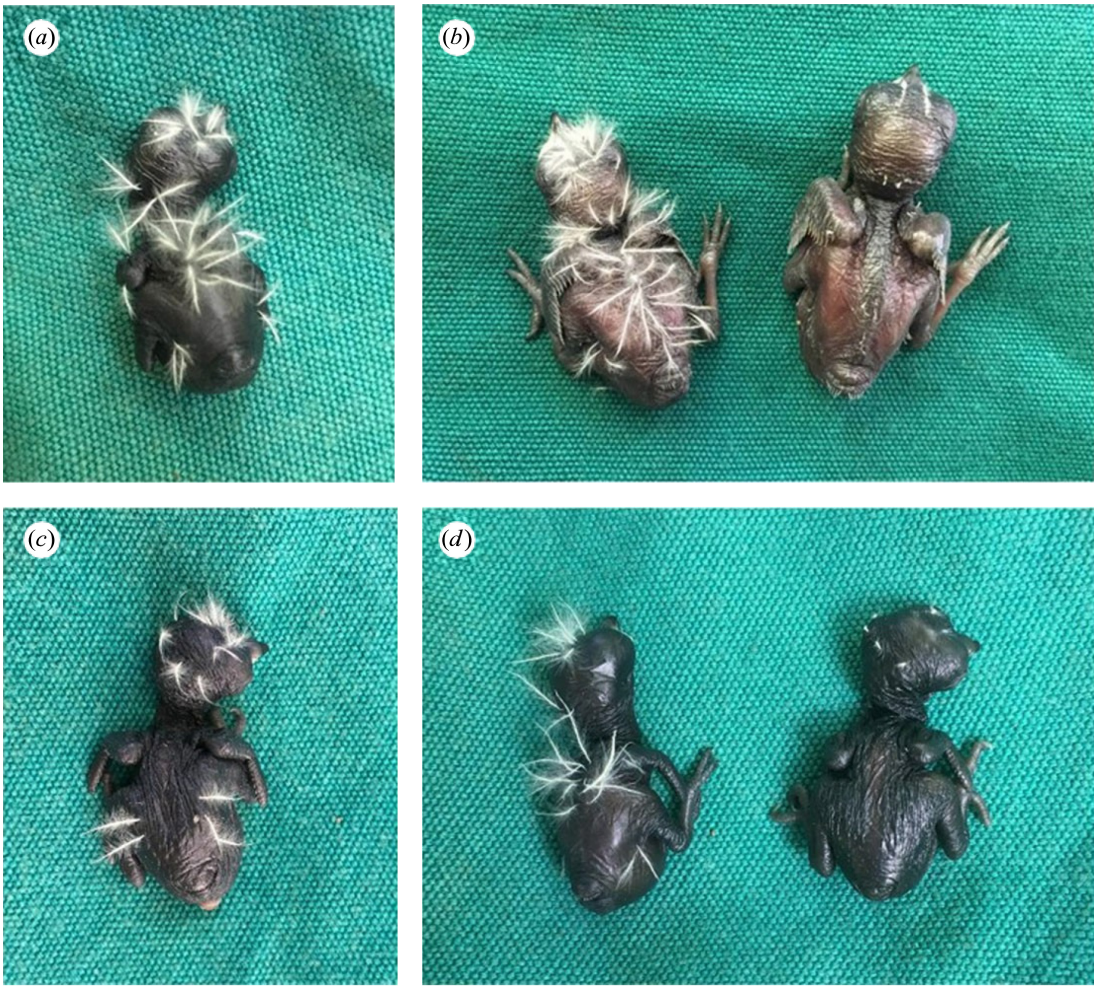

Figure 1. Photographs of large-billed gerygone and little bronze-cuckoo chicks. (*a*) An untrimmed gerygone on hatching day. (*b*) An experimental brood comprising one untrimmed host chick (left) and one trimmed host chick (right, both 3 days old). (*c*) An untrimmed cuckoo on hatching day. (*d*) An experimental brood comprising one untrimmed host chick (left) and one trimmed cuckoo chick (right, both on hatching day).

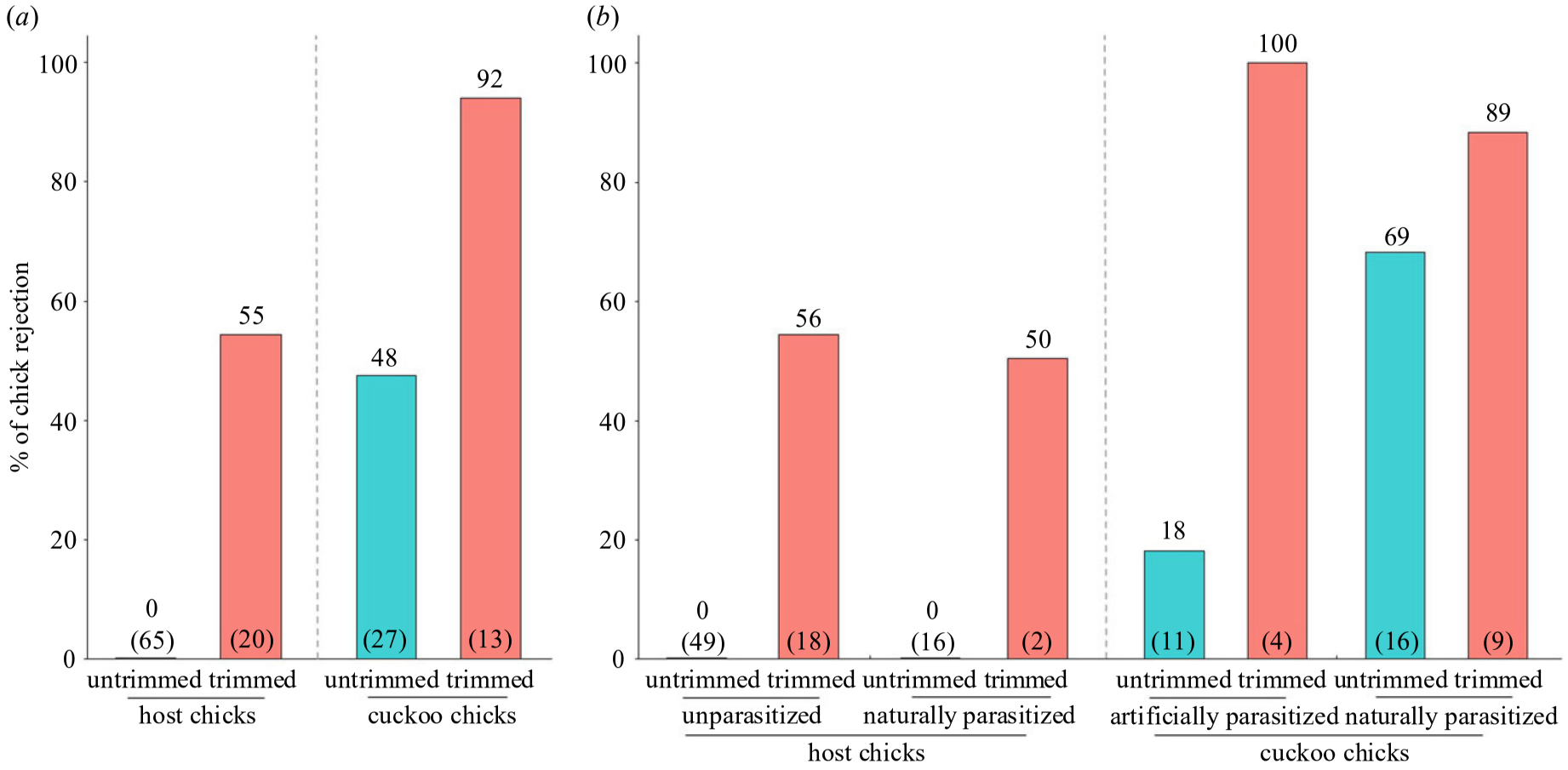

Figure 2. (*a*) The percentage of large-billed gerygone and little bronze-cuckoo chicks that were ejected according to whether down-feathers were trimmed. (*b*) The percentage of untrimmed and trimmed chicks that were ejected among host and cuckoo nestlings according to whether the nest was naturally or artificially parasitized (i.e. whether an adult cuckoo visited the nest during the egg-laying period). Sample sizes are given in parentheses at the base of the bar, and numbers above bars depict the exact percentage.

Discussion

Hosts that reject parasite nestlings may do so based either directly on chick phenotype (true recognition) or on recognition-free cues. True recognition is assumed to be maladaptive for cuckoo hosts if it relies on an imprinted template (Lotem 1993), and previous studies have found experimental support only for recognition-free mechanisms (Langmore et al. 2003, Grim 2007). Our results, however, provide the first experimental evidence that hosts can use true recognition when rejecting foreign nestlings, as large-billed gerygones regularly rejected nestlings that differed from their own offsprings' phenotype due to a lack of hatchling down-feathers. Gerygones combined this use of phenotypic cues with at least one additional chick-recognition-free cue, being more likely to reject cuckoo chicks when they had the opportunity to witness an adult cuckoo laying in the nest.

(a) Chick rejection based on true recognition

At least to the human observer, the number of down-feathers present on newly hatched chicks is the most obvious morphological cue available for discriminating between own and parasitic young; most host chicks have significantly more down-feathers than cuckoo chicks. Gerygones too were confirmed to use this cue in rejection decisions, being prompted to reject cuckoos, and even some of their own young, for which down-feathers were artificially removed. However, trimmed cuckoos were rejected at far higher rates than trimmed host young, indicating that gerygones use additional, as yet unidentified phenotypic cues. The begging calls of newly hatched chicks were audible to the human ear (H.-J.N. 2016, personal observation) and parents frequently made provisioning visits to the nest before they removed the chicks, so differences in begging call structure are a possible cue that warrants further investigation.

True recognition requires that hosts possess an internal template of the acceptable chick phenotype, to which they are able to compare cuckoo chicks. Given that to acquire this template solely through experience with a first brood would lead to maladaptively high rates of recognition error in the host of an evicting cuckoo (Lotem 1993), a gerygone's template must have an alternative origin. One possibility is that chick templates are largely innate, driven by strong selection for correct identification of own and parasitic young. Such innate templates could still be refined through experience, in much the

same way as songbirds have an innate template for their species's song that is refined through interactions with conspecifics (Langmore et al. 2009b). Rejection decisions can then be further refined through the complementary use of recognition-free cues (discussed below). The resulting recognition and rejection system is certainly effective for large-billed gerygones in our study area, as we never observed the mistaken rejection of host young (other than those that were trimmed). Notably, however, Sato et al. (2010) reported several cases of large-billed gerygones rejecting their own nestlings in a different study population, so it remains unclear whether low error rates are a general feature of gerygones' chick rejection behaviour. Recognition errors are most likely to occur in situations in which mimicry is highly accurate. In our study population, mimicry by little bronze-cuckoos was imperfect, because they had fewer nestling down-feathers than large-billed gerygone nestlings. However, host rejection was influenced more by the presence/absence of down-feathers than by the number of down-feathers per se. In addition, we found lower variation within than between broods in host down-feather abundance. This suggests that gerygones may be under selection for low intra-brood variation in the number of down-feathers to facilitate detection of cuckoo chicks, in much the same way as some other cuckoo hosts may experience selection for low intra-clutch variation in egg phenotype, facilitating detection of cuckoo eggs (Øien et al. 1995, Soler and Pape Møller 1996, Moksnes et al. 1999, Stokke et al. 2002). Such a process would require either that host and cuckoo chicks were present in the nest together (which occurs in a minority of nests) or that hosts remember their own chick morphology from previous broods.

Some combination of innate true recognition and more flexible mechanisms in gerygones' chick rejection would be consistent with our understanding of egg rejection mechanisms. Egg rejecter species show variation within and between populations in the form and extent of egg rejection behaviour (Soler et al. 1999, Moskát et al. 2010), and individual hosts' reactions towards foreign eggs may also vary with conditions or experience (Wang et al. 2015). The existence of both consistent and flexible patterns of egg rejection behaviour implies that both innate and learning mechanisms can be involved (Soler et al. 1999), and many host species appear to combine one or more variants of the true recognition process with proximate context-dependent factors when making rejection decisions (Moskát et al. 2010). Both egg rejection and chick rejection thus seem to be complex processes, using considerable mechanistic variation within and between species.

(b) Recognition-free discrimination

Gerygones were more than twice as likely to reject a cuckoo chick if an adult cuckoo had visited the nest during the egg-laying period than if the nest was parasitized artificially, indicating that the opportunity to observe or interact with a cuckoo at the nest strongly influenced rejection behaviour, as has also been observed in another bronze-cuckoo host (Langmore et al. 2009b). Moreover, our results showed the strongest effect of exposure to adult cuckoos in interaction with chick phenotype (table 1), suggesting that this cue on its own is not enough to prompt rejection and must be coupled with cues from the chicks themselves. This indicates that the combination of this contextual cue with one or more phenotypic cues may allow gerygones to substantially reduce the risk of mistakenly rejecting their own young, particularly given the accurate host–cuckoo nestling mimicry in this system (Reeve 1989, Wiley 1994, Sherman et al. 1997, Holen and Johnstone 2006).

Notably, if hosts use the presence of adult cuckoos as a cue to reject nestling cuckoos, the cue is ‘recognition-free’ with respect to chick phenotype, but does require the recognition of adult cuckoos. Based on behavioural responses, large-billed gerygones readily distinguish between adult cuckoos near their nests, which elicit mobbing, and predators or harmless species, which do not (F. Jacomb et al. 2015, unpublished data) (Mulyani 2004). Although it is unknown whether mobbing ever succeeds in preventing a cuckoo from laying, our results indicate that the recognition of adult cuckoos has an important role in gerygones' antiparasite defence, by increasing the accuracy of chick rejection decisions. Accordingly, our study provides support for strategy facilitation (Kilner and Langmore 2011), in which adaptations at one stage of the evolutionary arms race (in this case, the egg-laying stage) promote the evolution of defences at another stage (the nestling stage).

We found no evidence that large-billed gerygones use two other candidate recognition-free cues: hatch order or discordancy. A simple ‘reject the odd one out’ rule is useful only when there are multiple chicks in the nest, and only one of these is a cuckoo, a condition that is rarely met in large-billed gerygones owing to the shorter incubation period of cuckoo nestlings and the small clutch size of gerygones. A strategy of ‘reject the first hatched chick’ would, in theory, be relatively effective for gerygones in ridding themselves of cuckoos, particularly if enacted only when adult cuckoos have been seen

at the nest. However, the occurrence of multiple parasitism in this system (approx. 30% of all parasitized nests receive multiple cuckoos eggs (Brooker and Brooker 1989, Gloag et al. 2014)) reduces the benefit of such a rule of thumb substantially, because often another cuckoo will simply hatch to take the place of the rejected one.

(c) Implications for cuckoo–host coevolution and diversification in little bronze-cuckoos

Our results provide the first experimental demonstration that host defences can select for the evolution of nestling mimicry in a brood parasite. Previous work revealed that the nestlings of three bronze-cuckoo species are near perfect visual mimics of the host chicks they exploit (Langmore et al. 2011). Moreover, one host, the superb fairy-wren *Malurus cyaneus*, was less likely to reject cuckoo chicks of a species that specializes on fairy-wrens (Horsfield's bronze-cuckoo *C. basalis*) than a cuckoo species that uses fairy-wrens rarely (the shining bronze-cuckoo *Chalcites lucidus*) (Langmore et al. 2003). However, only recognition-free cues for chick discrimination were identified in this system, so it was unclear whether host rejection selected for mimicry of host young (Langmore et al. 2009b). Furthermore, some forms of chick mimicry might arise for reasons other than host rejection (Grim 2005), such as to exploit biases in host–parent communication and extract the optimal resources from host parents (Davies 2011). While it remains possible that the visual mimicry of little bronze-cuckoos also increases host provisioning rates, it seems likely that it has been primarily driven by gerygones' chick rejection behaviour.

In this study, our focus was demonstrating that true nestling recognition can evolve, contrary to the predictions of theory based on an imprinting model of chick rejection (Lotem 1993). Mis-imprinting constraints are not the only explanation, however, for the apparent scarcity of chick rejection across hosts of brood parasites. Effective rejection of cuckoo eggs can prevent the evolution of cuckoo chick rejection by making the cuckoo nestling a ‘rare enemy’, such that the benefits of discriminating against it are outweighed by the costs of recognition errors (Grim 2006, Britton et al. 2007, Dawkins 2016). Curiously, large-billed gerygones do not reject foreign eggs even though little bronze-cuckoo eggs look very different from their own. This is surprising given that hosts suffer fewer costs of parasitism by implementing defences early in the breeding cycle rather than later. Indeed, three non-mutually exclusive explanations for this are that (i) egg

rejection is constrained by poor visibility inside the nest, because dark-coloured bronze-cuckoo eggs are cryptic inside dark host nests (Langmore et al. 2009a, Gloag et al. 2014), (ii) egg rejection is constrained by bill morphology, because cuckoo eggs are too large or thick-shelled to be ejected and methods of egg rejection that remove or abandon whole clutches are too costly (Antonov et al. 2009, Rasmussen et al. 2010, De Mársico et al. 2013), and (iii) hosts may benefit by delaying rejection of the parasite until the chick stage when there is a risk of multiple parasitism, because allowing the cuckoo egg to remain in the nest reduces the probability that a host egg will be removed during subsequent parasitism events (the egg dilution hypothesis (Sato et al. 2010a, Gloag et al. 2014)).

Different subspecies of the little bronze-cuckoo exploit different hosts, and cuckoo mimicry of host nestlings can extend even down to the level of subspecies (Langmore et al. 2008). For example, *C. m. minutillus* mimics the dark skin and white down of nestling large-billed gerygones (Langmore et al. 2011), whereas *C. m. barnardi* mimics the pink skin and yellow down of the offspring of white-throated gerygones *Gerygone albogularis* (McGill and Goddard 1979, Grim 2005). In addition, the little bronze-cuckoo occupies a wider distribution and has more subspecies than any other *Chalcites* cuckoos (10 described subspecies, compared to just one to four variants of other bronze-cuckoos) (Payne and Sorensen 2005). Although the rejection behaviour of other little bronze-cuckoo hosts remains to be studied, it is plausible that the observed variation in little bronze-cuckoo chicks has evolved in response to true recognition and chick rejection by their hosts, ultimately reinforcing reproductive isolation among cuckoo populations that exploit different host species (Thompson 2005). Thus, unlike recognition-free mechanisms of chick rejection, true recognition of cuckoo chicks may have significant consequences for the coevolutionary trajectory of their parasites, by driving host-specific genetic diversification in parasite populations.

Ethics

The aim of this study was to explore the mechanism of chick rejection. While chick rejection by hosts leads to the death of the rejected chick, this experiment did not increase the frequency of chick death, because in a parasitized nest either the cuckoo chick or the host chicks always die under natural conditions. Our experimental manipulations may have influenced whether it was the cuckoo or the host chicks that died, but overall the experiments did not cause mortality in more nests than would happen naturally. All experiments were conducted under approval of the Australian National University Animal Experimentation Ethics Committee Protocol number A2016/16.

Footnotes

Electronic supplementary material is available online at https://dx.doi.org/10.6084/m9.figshare.c.4112972.

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Supplementary Material

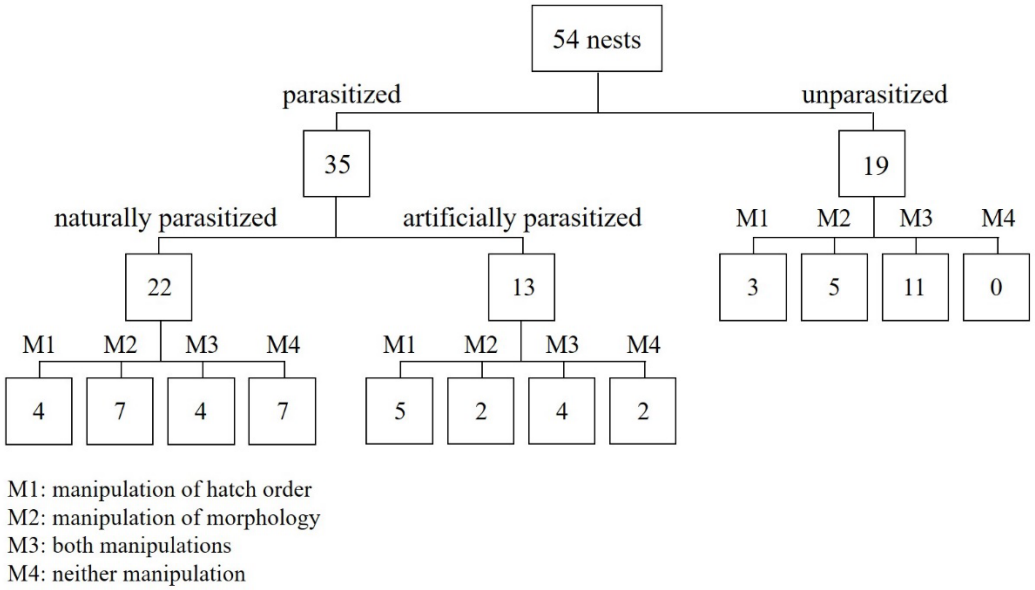

Figure S1. Flow diagram showing the design of the experiment. Numbers in boxes indicate the sample size of nests in the assigned treatments (M1-M4).

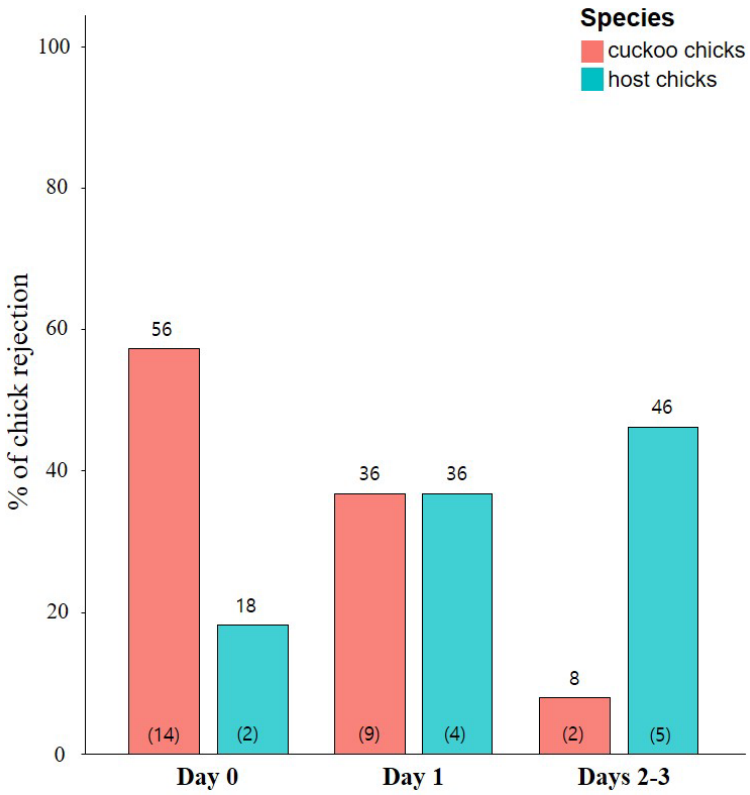

Figure S2. The percentage of cuckoo chicks and host chicks that were rejected according to the time to ejection (Day 0 = hatching day). Sample sizes are given in parentheses at the base of the bar, and numbers above bars depict the exact percentage.

Table S1-a. The procedure of a backward-elimination based on the Akaike Information Criterion (AIC) values to support the best-fit model with lowest AIC; see Table 1.

Model		AIC
Model1	species + trim + hatch order + exposure to adult cuckoo + discordancy + hatching date + species:trim + species:hatch order + trim:hatch order + species:exposure to adult cuckoo + trim:exposure to adult cuckoo + hatch order:exposure to adult cuckoo + species:discordancy + trim:discordancy + hatch order:discordancy + exposure to adult cuckoo:discordancy	96.83
Model2[a]	Model1 - trim:discordancy	94.15
Model3	Model2 - species: hatch order	94.15
Model4	Model3 - species: cuckoo visit to nest	88.93
Model5	Model4 - hatch order:discordancy	89.05
Model6	Model5 - species:discordancy	88.25
Model7	Model6 - exposure to adult cuckoo:discordancy	86.22
Model8	Model7 - trim:hatch order	84.66
Model9	Model8 - hatch order:exposure to adult cuckoo	83.26
Model10	Model9 - species:trim	82.79
Model11	Model10 - discordancy	80.76
Model12	Model11 - hatching date	79.81
Model13*	Model12 - hatch order	77.60
	(species + trim + exposure to adult cuckoo + trim:exposure to adult cuckoo)	
Model14	Model13 - trim:exposure cuckoo visit to nest	83.17
Model15	Model14 - exposure cuckoo visit to nest	83.14
Model17	Model13 - species	121.55
Model18	Model14 - trim	152.12
	(null model)	
Model13*	best-fit model with lowest AIC	

a. each variable (trim:discordancy) was removed each iteration (Model1)

Table S1-b. Models with Akaike's Information Criteria (AICc), measure of each model relative to the best model (Delta-AICc), Akaike's weight (AICc wi), and log likelihood

Model	df	AICc	Delta-AICc	AICc WT	Log-Likelihood	Evidence Ratio
Model 13	5	77.60	0.00	0.58	-33.55	
Model 12	6	79.81	2.21	0.19	-33.55	3.05
Model 11	7	80.76	3.17	0.12	-32.90	1.58
Model 10	8	82.79	5.19	0.04	-32.77	3
Model 9	9	83.26	5.66	0.03	-31.84	1.33
Model 8	10	84.66	7.06	0.02	-31.36	1.50
Model 7	11	86.22	8.62	0.02	-30.94	1
Model 6	12	88.25	10.65	0.00	-30.73	0
Model 4	14	88.93	11.33	0.00	-28.56	0
Model 5	13	89.05	11.45	0.00	-29.89	0
Model 3	15	91.52	13.92	0.00	-28.56	0
Model 2	16	94.15	16.55	0.00	-28.56	0
Model 1	17	96.83	19.23	0.00	-28.56	0
Null Model	1	152.12	74.52	0.00	-75.04	0

Table S2-a. The procedure of a backward-elimination based on the Akaike Information Criterion (AIC) values to develop the best-fit model with lowest AIC; see Table 2.

Model		AIC
Model1	species + trim + hatch order + exposure to adult cuckoo + discordancy + hatching date + species:trim + species:hatch order + trim:hatch order + species:exposure to adult cuckoo + trim:exposure to adult cuckoo + hatch order:exposure to adult cuckoo + species:discordancy + hatch order:discordancy + exposure to adult cuckoo:discordancy	90.13
Model2[a]	Model1 - species:exposure to adult cuckoo	84.27
Model3	Model2 - exposure to adult cuckoo:discordancy	78.91
Model4	Model3 - hatch order:discordancy	73.97
Model5	Model4 - trim:hatch order	70.66
Model6	Model5 - trim:exposure to adult cuckoo	66.71
Model7	Model6 - hatch order:cuckoo visit to nes	63.41
Model8	Model7 - species:trim	59.76
Model9	Model8 - species:discordancy	59.24
Model10	Model9 - species:hatch order	58.92
Model11	Model10 - hatch order	56.30
Model12	Model11 - trim	54.59
Model13	Model12 - hatching date	52.31
Model14	Model13 - exposure to adult cuckoo	51.33
Model	Model14 – discordancy (species)	50.11
Model15*	best-fit model with lowest AIC	

a. each variable (trim:discordancy) was removed each iteration (Model1)

Table S2-b. Models with Akaike's Information Criteria (AICc), measure of each model relative to the best model (Delta-AICc), Akaike's weight (AICc wi

Model	df	AICc	Delta-AICc	AICc WT	Log-Likelihood	Evidence Ratio
Model 15	2	50.11	0.00	0.45	-22.89	
Model 14	3	51.33	1.22	0.25	-22.89	1.8
Model 13	4	52.31	2.20	0.15	-21.57	1.67
Null Model	1	54.07	3.95	0.06	-27.03	2.50
Model 12	5	54.59	4.47	0.05	-21.38	1.20
Model 11	9	56.30	6.19	0.02	-20.84	2.50
Model 10	10	58.92	8.81	0.01	-20.65	2
Model 9	11	59.24	9.13	0.00	-19.22	0
Model 8	12	59.76	9.65	0.00	-17.78	0
Model 7	13	63.41	13.30	0.00	-17.78	0
Model 6	14	66.71	16.60	0.00	-17.47	0
Model 5	15	70.66	20.55	0.00	-17.33	0
Model 4	16	73.97	23.85	0.00	-16.70	0
Model 3	17	78.91	28.79	0.00	-16.70	0
Model 2	17	84.27	34.16	0.00	-16.70	0
Model 1	17	90.13	40.02	0.00	-16.70	0

Chapter 4.

Imperfect mimicry of host begging calls by a brood parasitic cuckoo and implications for host specialization

Hee-Jin Noh[1], Ros Gloag[2], Naomi E. Langmore[1]

[1] Research School of Biology, Australian National University, Building 116, Daley Road, Canberra, ACT 0200, Australia

[2] School of Life and Environmental Sciences, University of Sydney, Macleay Building A12, Science Road, Sydney, NSW 2006, Australia

Abstract

Coevolutionary interactions between avian brood parasites and their hosts often lead to the evolution of discrimination and rejection of parasite eggs or chicks by hosts based on visual cues, and the concomitant evolution of visual mimicry of host eggs or chicks by brood parasites. Hosts may also base rejection of brood parasite nestlings on vocal cues, which would in turn select for mimicry of host begging calls in brood parasite chicks. In cuckoos that exploit multiple hosts with different begging calls, call structure may be plastic, allowing nestlings to modify their calls to match those of their various hosts, or fixed, in which case we would predict either imperfect mimicry or divergence of the species into host specific lineages. In our study of the little bronze-cuckoo *Chalcites minutillus* and its primary host, the large-billed gerygone *Gerygone magnirostris*, we tested whether (a) hosts use nestling vocalisations as a potential cue to discriminate cuckoo chicks; (b) nestling little bronze-cuckoos mimic the begging calls of the large-billed gerygone throughout the nestling period; (c) the begging calls of little bronze-cuckoos are plastic, thereby facilitating mimicry of the calls of different hosts. Interestingly, the begging calls of little bronze-cuckoos were most similar to their gerygone hosts shortly after hatching (when rejection by hosts typically occurs) but became less similar as they get older. We found that not all begging calls produced by little bronze-cuckoo chicks matched those of large-billed gerygone on hatching day; the duration of calls made by rejected cuckoo chicks was significantly shorter than those produced by host chicks. These results are consistent with gerygone defences selecting for age-specific vocal mimicry in cuckoo chicks. We also found no evidence that little bronze-cuckoo begging calls were plastic. Cuckoo chicks that were cross-fostered as eggs to a naïve host with a different call type (lovely fairy-wren *Malurus amabilis*) did not change their begging calls to match those of this new host, but nor did their dissimilar begging call have any detrimental effect on their growth in this novel host.

Keywords

coevolution, brood parasitism, gerygone, bronze-cuckoo, begging call

Introduction

Obligate avian brood parasites such as cuckoos lay their eggs in the nests of other species, relying on the hosts to rear their offspring (Rothstein 1990). The interactions between avian brood parasites and their hosts often lead to the evolution of discrimination and rejection of parasite eggs or chicks by hosts (Davies 2000). In turn, this typically selects for the evolution of mimicry of host eggs or chicks by brood parasites (Soler 2014). If the mimetic traits are specific to a single host species, host races are more likely to evolve in the parasite, with each host race mimicking its respective host (Davies 2000, Avilés et al. 2006). Many hosts have evolved the ability to detect and eject eggs that differ in appearance from their own, selecting for highly refined, host-specific egg mimicry in brood parasites (Brooke and Davies 1988). Likewise, hosts may reject parasitic nestlings (Langmore et al. 2003, Grim 2006, Sato et al. 2010, Tokue and Ueda 2010, Noh et al. 2018), selecting for mimicry of host chicks in brood parasites (Langmore et al. 2011, De Mársico et al. 2012). A recent study demonstrated that one host uses visual cues to discriminate brood parasitic cuckoos from its own young and remove them from the nest (Noh et al. 2018), but it is unknown whether vocal cues also play a role in host rejection decisions.

According to coevolutionary theory, vocal mimicry of host nestlings by parasitic nestlings will evolve if hosts detect and discriminate against parasite young based on vocal cues (Langmore et al. 2003). Just as for visual mimicry, vocal mimicry of one host may constrain exploitation of additional hosts if the begging call does not resemble the calls of other host species. Unlike visual traits, however, vocal traits may exhibit a degree of plasticity (Jamie and Kilner 2017). Thus host discrimination of chick vocalisations may lead to two possible outcomes in parasites; (i) if begging call structure is genetically predetermined and inflexible, the evolution of mimicry of one host is likely to enforce a specialist parasitic strategy (either at the species-level, or via host-specific races within a generalist parasite), because exploitation of other host species may result in rejection of the parasitic chick based on its mis-matched call, or (ii), if begging call structure is plastic and can be modified to match those of the rearing host, this would allow exploitation of multiple host species. That is, phenotypic plasticity can generate similarity between parasite and host without exhibiting genetic specialization to any one host in particular (Langmore et al. 2008). An earlier study has demonstrated that begging calls are plastic and can be modified to match different hosts in a generalist cuckoo (Langmore et al. 2008), but this has not been investigated in a specialist species.

The little bronze-cuckoo is an obligate brood parasite that specialises on hosts of the genus *Gerygone*, primarily the large-billed gerygone *Gerygone magnirostris* in Australia. The gerygone hosts of the little bronze-cuckoo have the most sophisticated discrimination of brood parasite nestlings yet described for a cuckoo host, typically rejecting the nestling within hours of hatching (Sato et al. 2010) despite the high visual similarity of cuckoos to its own young (Langmore et al. 2011). Our previous study demonstrated that the large-billed gerygone discriminates the cuckoo chick from its own young using visual cues, but suggests that additional, as yet unidentified cues are also involved in discrimination (Noh et al. 2018). The begging call of newly-hatched chicks is one plausible cue that hosts may use to recognize cuckoo chicks. Discrimination of cuckoo chicks based on visual cues has selected for striking visual mimicry of host young by little bronze-cuckoos (Langmore et al. 2011, Langmore and Spottiswoode 2012), and likewise, cuckoos would be expected to evolve mimetic begging calls if hosts discriminate on the basis of vocal cues. Here, we tested whether (a) nestling vocalisations provide a potential cue for discrimination of parasitic young by hosts, by comparing the structure of cuckoo and host begging calls on the day of hatching, when discrimination occurs; (b) nestling little bronze-cuckoos mimic the begging calls of the large-billed gerygone throughout the nestling period; (c) the begging calls of little bronze-cuckoos are plastic, allowing exploitation of multiple hosts, or fixed, potentially constraining the cuckoo to a specialist strategy. We investigate vocal plasticity via a cross-fostering experiment in which cuckoos are reared from hatching by a naïve host (the lovely fairy-wren) that has a call that differs in structure from that of the cuckoo's primary host.

Methods

(a) Study species

The little bronze-cuckoo is widespread along the coast of northern and eastern of Australia. They are specialists on a single host genus (Gerygones), exploiting several species of *Gerygone* in Australia (Higgins 1999). The little bronze-cuckoo lays dark brown or olive-green eggs that are cryptic inside their dark nests, rather than mimetic (Brooker and Brooker 1989). The female cuckoo usually removes one host egg and lays

a single egg during the egg-laying period of their host (Langmore et al. 2009). The cuckoo chick hatches after about 15-17 days of incubation, one to two days before the host chicks, and ejects the host eggs or chicks from the nest within two days (Noh et al. 2018). The cuckoo chick fledges after 16-18 days. Hosts rarely reject cuckoo eggs (Gloag et al. 2014), but if hosts recognize the cuckoo chick, they reject it within a few hours of hatching (Sato et al. 2010, Tokue and Ueda 2010). At our study site, 69% of cuckoo chicks were rejected (Noh et al. 2018). This chick rejection has selected for highly accurate visual chick mimicry by the cuckoos (Langmore et al. 2011, Noh et al. 2018).

To test whether little bronze-cuckoos modify their begging calls in the nest of a different host, we cross-fostered cuckoo eggs to the nests of lovely fairy-wrens *Malurus amabilis*. Lovely fairy-wrens are an insectivorous passerine of similar size to large-billed gerygones that are endemic to Cape York Peninsula, Queensland. They are rare hosts of the brush cuckoo *Cacomantis variolosus* (De Geest and Leitão 2017), but no parasitism by the little bronze-cuckoo has been reported. They have an incubation period of 12-16 days, and a nestling period of 12-14 days (Leitão et al. 2019).

(b) Study site and field methods

The study was conducted during the breeding season of the little bronze-cuckoo in Cairns (Aug - Dec 2017-2019) and Lockhart River (July 2018), Queensland. In these areas, little bronze-cuckoos parasitize large-billed gerygones and fairy gerygones *Gerygone palpebrosa*. Large-billed gerygones build their nests along tidal or fresh water creeks, and fairy gerygones build nests in dense mangrove and at forest edges (Higgins 1999). The habitats of lovely fairy-wrens include rainforest edge, woodland, and mangroves (Leitão, et al. 2019). The nests of gerygones and lovely fairy-wrens were located by daily searching and monitored subsequently with nest checks at least every 3-4 days (gerygones: 16°55′ S, 145°46′ E and 12°37'S 143°25'E, and lovely fairy-wrens: 16°55′ S, 145°46′ E).

(c) Begging call recordings

We recorded the begging calls of large-billed gerygones and little bronze-cuckoos on day 0 (= hatching day), day 3±1, day 7±2 and day 13±3 in 2016-2019. We also recorded

the begging calls of fairy gerygones and lovely fairy-wrens on day 3±1, day 7±2 and day 13±3 in 2016-2018. Insufficient fairy gerygone nests were found to include them in statistical analyses, but recordings were made at two nests and sonograms of these are included. Calls were recorded using a Sony tie-clip miniature microphone (ECM T6) and a recorder (TASCAM DR-05). The microphone was clipped to the back of the nests. All the nests were monitored from a hide or filmed using video cameras (Panasonic VX870M) to confirm that recordings did not disrupt provisioning by parents and to capture instances of chick rejection by host parents. Video cameras were placed approximately 5-7m from the nests.

(d) Cross-fostering experiments and measurements

To determine whether begging calls produced by cross-fostered little bronze-cuckoos in the nests of lovely fairy-wrens were sufficient to stimulate adequate provisioning by the foster parents, we compared growth rates of cross-fostered cuckoos to those of cuckoos reared by their natural host, the large-billed gerygone. Cross-fostering experiments were conducted in 2017 and 2018. Since the incubation period of cuckoo eggs is longer than that of lovely-fairy wren eggs, cuckoo eggs were pre-incubated in large-billed gerygone nests where they had been originally laid, and then transferred to lovely fairy-wren nests so that the cuckoo chicks hatched 1-3 days before the fairy-wren chicks were due to hatch (as typically happens in the nests of their biological hosts).

In total, 24 cuckoo eggs (18 in 2017 and 6 in 2018) were swapped to the nests of lovely fairy-wrens, and all were accepted by their hosts. Ten nests (9 in 2017 and 1 in 2018) were depredated at the egg stage, and 14 nests survived to hatching (9 in 2017 and 5 in 2018). Of the 14 cuckoo nestlings, 12 (8 in 2017 and 4 in 2018) were depredated in the nest, and only 2 fledged (1 in 2017 and 1 in 2018). For comparison, we also monitored 28 large-billed gerygone nests that were naturally parasitized by little bronze-cuckoos in 2017. We measured the weight of all chicks at four developmental stages (day 0 = hatching day, day 3±2, day 7±2, day 13±2).

(e) Begging call analysis

From each recording, we analysed five begging calls recorded from nestlings while parents were brooding or when parents visited for provisioning. Six acoustic features for each call (a note) were measured; call duration (sec), high frequency (kHz), low

frequency (kHz), peak frequency (kHz), frequency bandwidth (kHz), and the difference in frequency between the beginning and the end of the call. All measurements of begging calls were conducted using RavenPro sound analysis software (Version 1.5, Cornell Laboratory of Ornithology).

(f) Statistical analysis

Analysis of acoustic variables was conducted using discriminant function analysis in JMP (v. 6.0). We first performed a discriminant function analysis using a stepwise procedure to assess if this analysis could separate the calls of accepted cuckoos, rejected cuckoos, and hosts on hatching day. We also used pairwise comparisons to test the same dataset for multiple comparisons between group levels for each call variable using R (ver. 3.4.4).

Second, we used a discriminant function analysis to test whether the model could distinguish between the begging calls of the four species (cuckoo chicks reared by large-billed gerygones, cuckoo chicks reared by lovely fairy-wrens, large-billed gerygone chicks and lovely fairy-wren chicks). We also used linear mixed models (function lmer in R, library lmerTest, Bates et al. 2011) with fixed effects of species, age, and the interaction of group and age to compare the begging calls of 1) cuckoo and large-billed gerygone nestling and 2) cuckoos reared by different species. A nest identifier was included as a random effect because calls from multiple chicks in a nest were recorded.

Finally, to test whether cuckoo chicks suffered a growth cost when they were reared by a different species, we used a linear mixed model. A previous study showed that a third order polynomial provides a good fit to the growth patterns of bronze-cuckoos from hatching day to 13 days old (Medina et al. 2019), so we fitted a third order polynomial for the growth model. Cuckoo weight was used as the response variable, and we include chick age, host species, year, the number of times the chick was handled, and the interaction between age and host species as fixed effects. Each cuckoo chick was measured multiple times, so a nest identifier was included as a random effect. In addition, to complement this analysis, we calculated the residuals of each data point from the average growth curve (Anderson et al. 2009). Positive residual values designate better growth performance of an individual chick compared to the average and vice versa. We used the residuals in a linear mixed model to explore whether host species could explain

variation in the residuals. We used the residuals as the response variable and we included host species, year, and the number of times the chick was handled as fixed variables, and nest ID as a random effect. These analyses were conducted using R (ver. 3.4.4) and the lmerTest and emmeans package.

Results

(a) Do nestling vocalisations provide a potential cue for discrimination by host?

The begging calls of little bronze-cuckoos generally resembled those of large-billed gerygones on hatching day (no difference in high frequency (kHz), low frequency (kHz), peak frequency (kHz), frequency bandwidth (kHz), and the difference in frequency between the beginning and the end of the call among rejected cuckoos, accepted cuckoos, and hosts nestlings, all $p > 0.05$, Figure 1). However, there was one notable exception; the duration of calls made by rejected cuckoo chicks was significantly shorter than those made by host chicks ($p = 0.03$, Figure 1). The call duration of accepted and rejected cuckoo nestlings did not differ significantly ($p = 0.79$, Figure 1), even though accepted cuckoo chicks made marginally shorter calls than those made by host chicks ($p = 0.08$, Figure 1). Therefore, the calls of accepted cuckoo chicks were intermediate between those of host chicks and rejected cuckoo chicks. We do not have enough data for statistical analysis of the begging calls of the fairy gerygone, but those that were recorded were shorter than those of large-billed gerygones (Figure 2).

(b) Do little bronze-cuckoo nestlings mimic the begging calls of the large-billed gerygone?

Visual inspection of sonograms showed that the begging calls of little bronze-cuckoos are a close match to the begging calls of large-billed gerygone on hatching day, but become increasingly different as they get older (Figure 3). Similarly, a discriminant function analysis failed to discriminate between little bronze-cuckoo and large-billed gerygone begging calls on hatching day, but could distinguish between their calls based on all call variables except frequency bandwidth at later ages (day 7 and day 13, Figure 4).

There were some significant differences in all call measurements between cuckoo nestlings and large-billed gerygone nestlings at all three ages (Table 1 and 2, Figure 1-b), and three call variables (call duration, frequency bandwidth, and maximum frequency) showed significant changes with age (Table 2, part a). We also found a significant interaction between host species and nestling age for call duration, maximum frequency and differences in frequency (Table 2, part a), indicating that the changes in call structure with age differ between large-billed gerygones and little bronze-cuckoos.

(c) Do cuckoo chicks modify their begging calls when cross-fostered to a different host?

The begging calls of lovely fairy-wrens were significantly different from those of large-billed gerygones (Figure 5 and Table 1). However, the begging calls of cuckoos reared by lovely fairy-wrens did not differ significantly from those reared by large-billed gerygones at any of the three stages of the nestling period (Figure 5). Consistent with these results, we found no significant effect of host species and no interaction between host species and nestling age for any call variable (Table 2, part b). However, there was a significant effect of nestling age for call duration, frequency bandwidth, and maximum frequency (Table 2, part b). These results indicate that some call variables changed as cuckoos grew up, but the change in the call variables with nestling age did not differ depending on which host reared the cuckoo.

Growth patterns of cuckoo nestlings did not differ significantly according to which host they were reared by ($F_{1,38}$ = 1.32, p = 0.26, Figure 6-a). There was no significant difference in growth rates in 2017 versus 2018 ($F_{1,39}$ = 2.38, p = 0.13), or in relation to the number of times the chick was handled ($F_{1,48}$ = 1.48, p = 0.23). We found the same results when using residuals from the average curve ($F_{1,38}$ = 0.89, p = 0.35, Figure 6-b). Again, neither year ($F_{1,40}$ = 2.20, p = 0.15) nor the number of times the chick was handled ($F_{1,42}$ = 0.976, p = 0.33) had a significant effect on chick growth.

Figure 1. (a) Mean ± s.e. duration (seconds) of begging calls produced by large-billed gerygone chicks (*n* = 7), accepted cuckoo chicks (*n* = 4), and rejected cuckoo chicks (*n* = 3) on hatching day. (b) Sonograms of nestling begging calls for large-billed gerygones chicks, accepted cuckoo chicks, and rejected cuckoo chicks.

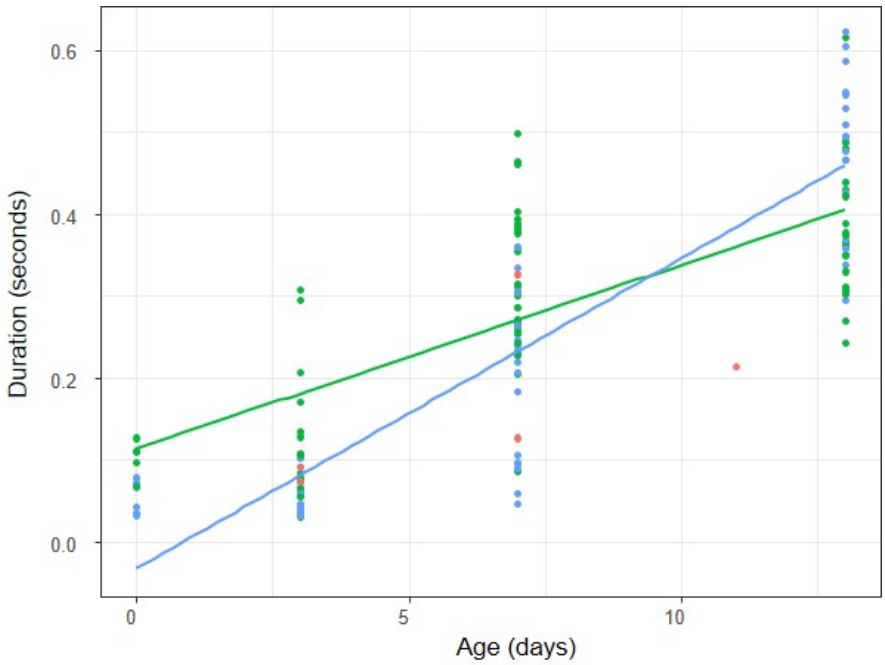

Figure 2. Duration (seconds) of begging calls in relation to age of large-billed gerygones (green, *n* = 36 chicks), little bronze-cuckoos (blue, *n* = 38), and fairy gerygones (red, *n* = 2).

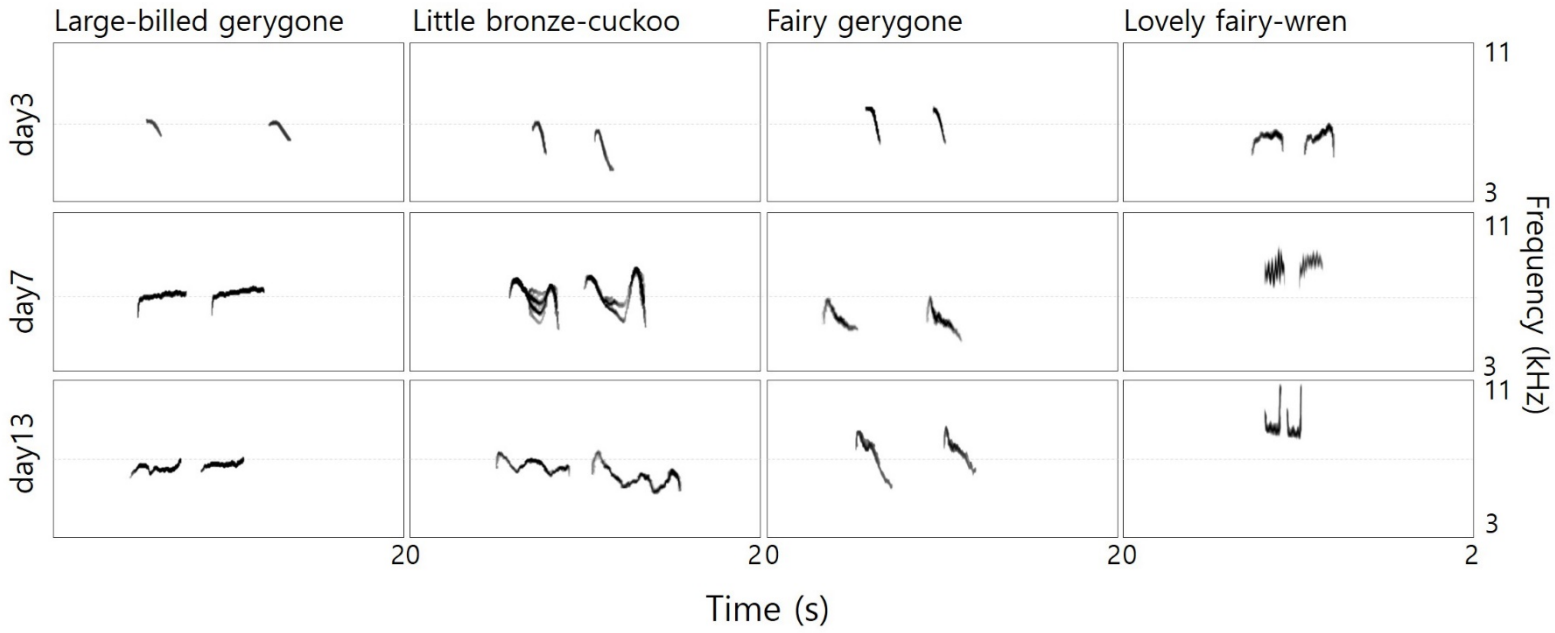

Figure 3. Sonograms of nestling begging calls for large-billed gerygones, little bronze-cuckoos, fairy gerygones and lovely fairy-wrens at three different ages during the nestling period.

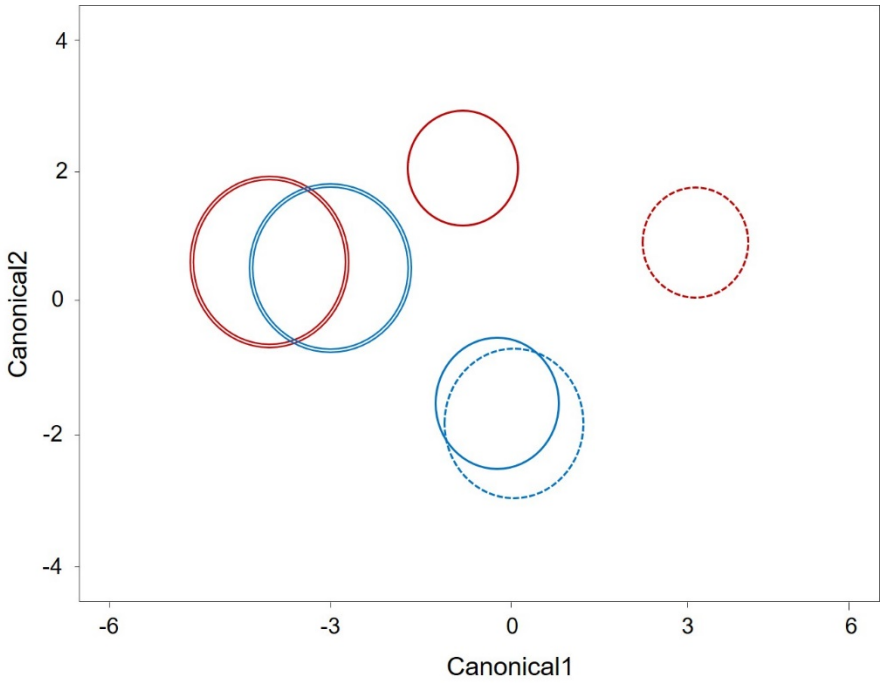

Figure 4. Canonical plots from discriminant function analysis based on the seven begging call measurements from large-billed gerygone chicks (blue, *n* = 37), and little bronze-cuckoo chicks reared by large-billed gerygones (red, *n* = 53) at three different nestling stages, day 0 (double solid line) and day 7 (single solid line), and day 13 (dotted line). Discriminant function analysis labels each multivariate mean with a circle. The size of the circle corresponds to a 95% confidence limit for the mean. Groups that are significantly different have non-intersecting circles.

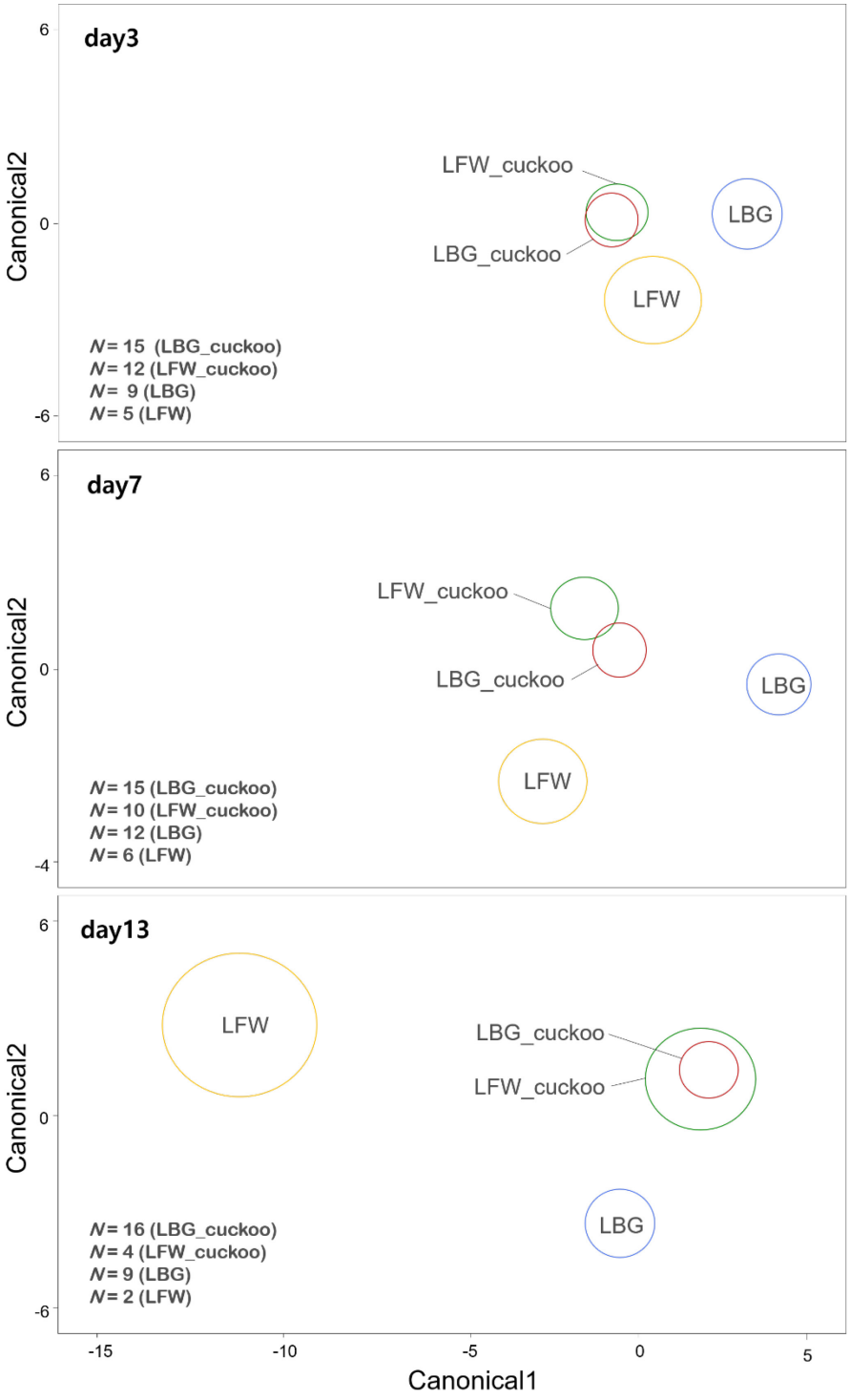

Figure 5. Canonical plots from discriminant function analysis based on the seven begging call measurements from large-billed gerygone chicks (LBG, blue), lovely fairy-wren chicks (LFW, yellow), and little bronze-cuckoo chicks reared by large-billed gerygones (LBG_cuckoo, red) and lovely fairy-wrens (LFW_cuckoo, green) at three different ages during the nestling period. Discriminant function analysis labels each multivariate mean with a circle. The size of the circle corresponds to a 95% confidence limit for the mean. Groups that are significantly different have nonintersecting circles.

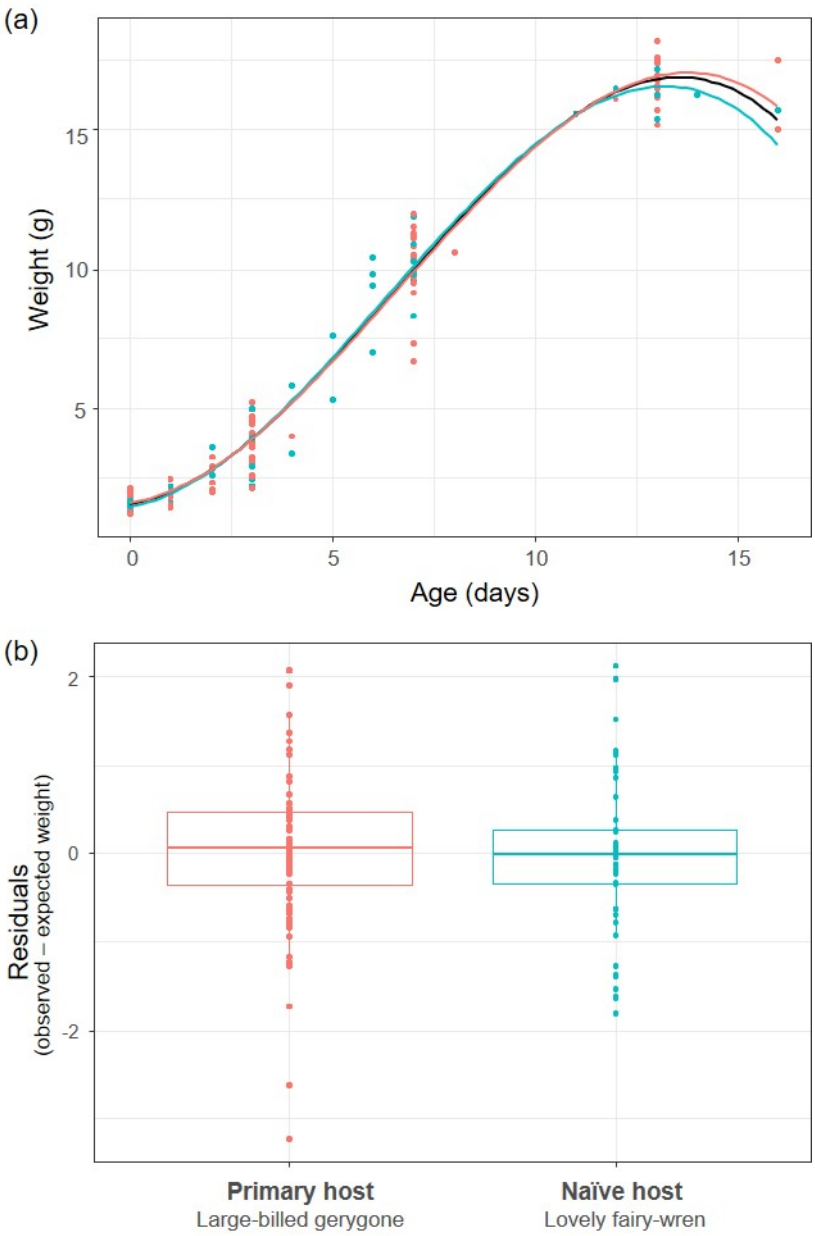

Figure 6. (a) Growth of little bronze-cuckoo chicks in large-billed gerygone nests (primary host, *n* = 28, red points and line) and lovely fairy-wren nests (naïve host, *n* = 14, blue points and line). The average growth curve from all datapoints is shown in black. (b) Boxplot of residuals of each data point from the average growth curve.

Table 1. Discriminant function analysis of the begging calls of cuckoo chicks reared by large-billed gerygone and cuckoo chicks cross-fostered lovely fairy-wren, large-billed gerygone chicks, and lovely fairy-wren chicks.

Nestling age	Significant variables	Wilk's λ	Exact *F*	*p*
Day 3	Call duration	<.0001	3.30	**<.0001**
	Differences in frequency			
Day 7	Call duration	<.0001	7.57	**<.0001**
	Maximum frequency			
	Minimum frequency			
	Peak frequency			
Day 13	Call duration	<.0001	12.53	**<.0001**
	Maximum frequency			
	Minimum frequency			
	Differences in frequency			

Table 2. Comparison of age-matched begging calls between (a) cuckoo nestlings reared by large-billed gerygones (LBG_cuckoo) vs large-billed gerygone (LBG) nestlings and (b) cuckoos reared by LBG vs cuckoo nestlings reared by a non-host, the lovely fairy-wren (LFW_cuckoo)

	Group		Age		Group × Age	
	Estimate (s.e)	*p*	Estimate (s.e.)	*p*	Estimate (s.e.)	*p*
(a) cuckoo (LBG_cuckoo) vs host (LBG)						
Call duration	0.13 (0.03)	**< 0.001**	0.04 (0.002)	**< 0.001**	-0.02 (0.004)	**< 0.001**
Frequency bandwidth	-256.69 (78.65)	**0.002**	-18.62 (6.08)	**0.003**	3.91 (9.76)	0.69
Maximum frequency	470.32 (228.48)	**0.04**	44.52 (16.75)	**0.01**	-54.69 (27.22)	**0.04**
Minimum frequency	731.74 (196.60)	**< 0.001**	2.20 (14.81)	0.88	26.18 (23.96)	0.28
Peak frequency	533.31 (208.17)	**0.01**	9.90 (15.56)	0.53	-27.25 (25.215)	0.28
Differences in frequency	-743.39 (252.56)	**< 0.001**	2.96 (19.44)	0.88	-82.78 (31.24)	**< 0.001**
(b) cuckoo in primary host (LBG_cuckoo) vs cuckoo in naïve host (LFW_cuckoo)						
Call duration	0.01 (0.03)	0.71	0.04 (0.002)	**<0.001**	-0.0006 (0.004)	0.15
Frequency bandwidth	93.87 (136.86)	0.49	-18.62 (8.80)	**0.04**	4.86 (18.74)	0.80
Maximum frequency	92.63 (300.30)	0.76	43.54 (18.40)	**0.02**	-14.91 (38.33)	0.70
Minimum frequency	-47.89 (266.74)	0.86	-1.17 (17.15)	0.95	-44.97 (36.52)	0.22
Peak frequency	69.48 (267.76)	0.80	8.32 (16.91)	0.63	-9.96 (35.80)	0.78
Differences in frequency	89.98 (359.49)	0.80	2.85 (22.78)	0.90	31.89 (48.29)	0.51

Discussion

Little bronze-cuckoos produce a begging call that resembles that of their large-billed gerygone host on hatching day, but becomes increasingly dissimilar throughout the nestling period. This may represent a period of strong selection for mimicry during the period when rejection of cuckoo chicks by hosts occurs (within 2 days of hatching), followed by a relaxation of selection for mimicry at later stages of the nestling period, when cuckoo nestlings were never rejected by their hosts. Calls may still be under selection for mimicry later in the nestling period in order to stimulate sufficient provisioning by the foster parents, so as to "tune into" host parental provisioning rules (Davies 2011). However, studies in other species have shown that in order to stimulate adequate provisioning from hosts, parasite begging calls must resemble host begging calls in some, but not all, features of call structure (Madden and Davies 2006) and that call structures that differ considerably in per-unit structure from that of host young may nevertheless be highly efficient at eliciting provisioning by hosts (Davies et al. 1996, Gloag and Kacelnik 2013).

On hatching day (the day on which rejection by hosts typically occurs), the begging calls of cuckoo chicks that were rejected by their hosts were significantly less similar to the calls of host nestlings than were the calls of cuckoo chicks that were accepted by the host. Specifically, the duration of the begging calls of rejected cuckoo chicks was significantly shorter than the duration of host begging calls, whereas the duration of the begging calls of accepted cuckoo chicks did not differ significantly from the duration of host begging calls. This result indicates that call duration on hatching day may be a cue that is used by hosts to discriminate parasite chicks. This evidence of host discrimination based on call structure suggests that begging call mimicry in little bronze-cuckoos has evolved in response to host discrimination based on acoustic cues. Our finding extends our understanding of how hosts of the little bronze-cuckoo discriminate cuckoo chicks from their own young, despite striking visual mimicry of host nestlings by cuckoos (Langmore et al. 2011). Previous work revealed that large-billed gerygones recognize cuckoo chicks in part by the density of nestling down feathers on the chicks; cuckoo nestling down is sparser and finer than host down feathers (Noh et al. 2018). However, when feathers were removed from nestling cuckoo and host chicks, the trimmed cuckoos were rejected at a higher rate than the trimmed host young, indicating that gerygones use an additional and unidentified cue to discriminate foreign chicks (Noh et al. 2018). The results of this study suggested the possibility that the additional cue used by large-

billed gerygones is begging call duration, indicating that gerygones combine several sources of information to reduce the risk of rejection error.

Although the resemblance between cuckoo and large-billed gerygone calls was greatest on hatching day, they still differed in one dimension, call duration; cuckoos produced shorter calls than large-billed gerygones (Figure 2). A possible explanation for the shorter begging calls of newly hatched cuckoo nestlings is that a second host of little bronze-cuckoos in Queensland, the fairy gerygone, might produce calls of shorter duration, selecting for intermediate calls in cuckoo chicks to facilitate exploitation of multiple species in the *Gerygone* genus. Imperfect mimicry for this purpose has been identified in the egg colour (Feeney et al. 2014) and the nestling skin colour (Langmore et al. 2011) of another species of bronze-cuckoo. While we don't yet have sufficient data from the various other gerygone hosts of little-bronze cuckoos to test this idea, our preliminary data show that fairy gerygone chicks do produce shorter begging calls than those of large-billed gerygone chicks (Figure 2).

Our cross-fostering experiments demonstrated that little bronze-cuckoos retained the same begging call structure when they were cross-fostered to a host with a different begging call, the lovely fairy-wren. This differs from a study of the congeneric Horsfield's bronze-cuckoo *Chalcites basalis*, which altered its begging call when cross-fostered to a different host (Langmore et al. 2008). A possible explanation for this result relates to the different strategies of parasitism of the two species; the little bronze-cuckoo is a specialist that exploits *Gerygone* hosts only, whereas the Horsfield's bronze-cuckoo is a generalist that exploits several different genera (Brooker and Brooker 1989). The hosts of Horsfield's bronze-cuckoos therefore show greater diversity in begging call structure than those of little bronze-cuckoos (Langmore et al. 2008), and Horsfield's bronze-cuckoos may be under greater selection from hosts to produce a plastic begging call that can be modified to match those of its various hosts. Conversely, in the specialist little bronze-cuckoo it may be too costly to acquire the appropriate begging calls, given that plasticity requires repeated sampling of environmental cues to be accurate, and little bronze-cuckoos are specialist parasites (Jamie and Kilner 2017).

An alternative explanation for the lack of modification of little bronze-cuckoo begging calls in the nest of a new host is that this particular new host failed to provide a trigger for call modification. The proposed mechanism underlying call modification in Horsfield's bronze-cuckoos was that a non-mimetic begging call failed to stimulate provisioning by the host, triggering a change in call structure in the cuckoo chick (Langmore et al. 2008). Our results showed that the nestling growth patterns of cuckoos reared by large-billed gerygones and lovely fairy-wrens did not differ, suggesting that lovely fairy-wrens did not reduce provisioning in response to a non-mimetic call (Figure 6). Lovely fairy-wrens may have maintained an adequate provisioning rate when faced with a cuckoo chick because either a) they respond to the same cues as large-billed gerygones (for example, they might provide more food with increasing call rate, regardless of call structure), or b) they are not a primary host of any cuckoo species, so they have not evolved discrimination based on call structure. This may suggest that the cuckoo begging call itself effectively stimulated provisioning in the nests of a non-host species, the lovely fairy-wren. However, this does not preclude the possibility that their begging calls are plastic, because it is possible that little bronze-cuckoo chicks are able to modify their begging calls if this is triggered by reduced provisioning by the host (Langmore et al. 2008). This possibility requires further investigation, by cross-fostering little bronze-cuckoos to a secondary host species that has a different begging call structure from large-billed gerygones and shows reduced provisioning in response to a non-mimetic begging call.

Ethics

All experiments were conducted under approval of the Australian National University Experimentation Ethics Committee Protocol number A201539 and A2016/16.

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Synthesis & Conclusions

Host defences against brood parasitic cuckoos differ in both type and strength across populations and species. Some hosts have no defence against parasitic cuckoos, and at the other extreme, where a single defensive line is not sufficiently robust to deter the parasite completely, others have developed multiple defences in the same and/or different stages of the breeding cycle. The host defences are interdependent; an adaptation at one stage can facilitate or block the defences at another stage (Britton et al. 2007, Kilner and Langmore 2011). Therefore, to understand the outcomes of coevolutionary arms races between hosts and cuckoos, it is important to study adaptations and counter-adaptations across all stages of the breeding cycle.

Frontline defences have the greatest potential to minimize the costs of parasitism to hosts, because unlike egg and chick stage defences, the host's own reproductive attempt can remain entirely intact (Feeney et al. 2012). My results suggest that cuckoo parasitism has selected for a portfolio of frontline defences against cuckoos in large-billed gerygones. Although frontline defences appeared to be largely ineffective in themselves, they facilitated defences at the chick stage (by increasing the accuracy of chick rejection), thereby providing a rare example of strategy facilitation in brood parasite hosts (Kilner and Langmore 2011).

Defences at the egg stage of the breeding cycle are arguably the most common type of defence amongst brood parasite hosts. The evolution of egg rejection by hosts and consequent selection for egg mimicry by cuckoos constitute the most well-studied example of a coevolutionary arms race between avian brood parasites and their hosts. Unlike most brood parasite hosts, large-billed gerygones typically accepted cuckoo eggs in their nests. However, under some circumstances they abandoned nests containing cuckoo eggs. Our results demonstrate that large-billed gerygones make adaptive decisions about whether to abandon or accept cuckoo eggs; cuckoo eggs were accepted when their presence reduced the chance of loss of host eggs during subsequent parasitism events, and were abandoned only when there was no possibility of rearing their own chicks. This result provides strong support for the 'Clutch dilution hypothesis' (Sato et al. 2010) and demonstrates a sophisticated level of plasticity in host decision making.

The benefits of cuckoo egg acceptance appear to have selected for postponement of rejection of the parasite until the nestling stage. However, theory predicts that nestling rejection (using true recognition) is maladaptive for cuckoo hosts; if hosts learn the appearance of their own chicks through imprinting on their first brood, a host parasitized during its first breeding attempt would falsely imprint on the lone foreign chick as its own young and thereafter reject its own offspring for the rest of its life (Lotem 1993). However, my results challenge this hypothesis and demonstrate for the first time that hosts can evolve the ability to discriminate cuckoo chicks using a 'recognition cue' (Noh et al. 2018, Attisano et al. 2018). This true recognition has, in turn, selected for striking visual mimicry of host young by little bronze-cuckoo chicks (Langmore et al. 2011, Tanaka 2016).

Even if there are strong selective pressure on both hosts and brood parasites, it is expected that the selective pressure will be stronger in brood parasites than their hosts; a cuckoo which fails to outwit its hosts will never reproduce, whereas a host that fails to outwit cuckoos can still produce young because some host nests are not parasitized (Dawkins and Krebs 1979). While the parasitism strategies of the generalist cowbirds, especially the shiny cowbirds, have been well-studied, the strategies of specialist cuckoo have received less attention. My results show that specialist little bronze-cuckoos produce a similar begging call to their gerygone hosts on hatching day, when rejection by hosts typically occurs, but it is less similar at later stages of the nestling period, indicating a relaxation of selection for mimicry. On hatching day (when rejection by hosts typically occurs), the begging calls of cuckoo chicks that were rejected by the host parents were significantly less similar to the calls of host nestlings than were the calls of cuckoo chicks that were accepted by the hosts. We found no evidence that little bronze-cuckoo begging calls were plastic; little bronze-cuckoos retained the same begging calls structure when cross-fostered to a naïve host species with a different begging call. These results suggest that large-billed gerygones may use begging call structure as a cue to discriminate parasitic young and that this has selected for increased similarity of parasite begging calls to host calls during the early nestling period. Functional olfactory systems in birds is an area of increasing research (Roper, 1999, Hagelin and Jones, 2007) For future study, therefore, consideration should be given to the possibility of olfactory and chemical mechanisms for detection of cuckoo chicks by hosts. Specifically, the odour on the cuckoo eggs and chicks derived from preen gland secretions may allow hosts to detect the foreign imposters and in turn, parasites may evolve olfactory mimicry of host species (Sealy and Underwood 2012.).

In conclusion, the results of my thesis demonstrate that large-billed gerygones exhibit a facultative response to cuckoo parasitism, switching between rejection of cuckoo eggs and cuckoo chicks so as to maximize the survival of their young. Moreover, my results suggest that adaptations at one stage of the evolutionary arms race can promote the evolution of defences at another stage, highlighting the importance of studying defences at all stages of the breeding cycle to fully understand the coevolutionary interactions between brood parasites and their hosts.

Future directions

Species interactions can be powerful generators of evolutionary diversification (Ehrlich and Raven 1964, Yoder and Nuismer 2010). Avian brood parasitism is an interspecific interaction that has been proposed as a model system for the study of adaptation and coevolution (Rothstein 1990), and use of different hosts can exert divergent selection for parasitic specialization resulting in multiple host specific races or eventually speciation (Davies 2010). Evidence that the evolution of host defences may ultimately select for divergence of a cuckoo species into several host-specific races or new species comes from a comparative analysis, which demonstrated that parasitic cuckoos have higher speciation rates than closely related non-parasitic cuckoos, and cuckoo species with more hosts have more recognized subspecies (Krüger et al. 2009). However, a recent comparative study indicated that coevolution may promote phenotypic diversification amongst avian brood parasites, but not speciation (Medina et al. 2016). Thus, the question of whether coevolution drives speciation is currently highly contentious in the literature.

In evolutionary processes, the extent of gene flow among populations determines their potential for genetic differentiation (Spottiswoode et al. 2011). If coevolution between cuckoos and hosts promotes genetic diversification of cuckoos, we would predict that there may be some degree of assortative mating leading to genetic divergence between little bronze-cuckoos that exploit different hosts. There is some indirect evidence in support of this possibility, because a study investigating the phylogeographic structure of the little bronze-cuckoo found some, incomplete differentiation between the sympatric subspecies *C. m. minutillus* and *C. m. russatus* (Joseph et al. 2011). However, this study

was conducted on museum specimens, so the identity of the host species was unknown for these specimens. Future studies are needed to test whether there is a relationship between host specificity and patterns of genetic diversity.

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Appendix

PROCEEDINGS B

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Research

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Author for correspondence:
Hee-Jin Noh
e-mail: heejin.noh@anu.edu.au

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True recognition of nestlings by hosts selects for mimetic cuckoo chicks

Hee-Jin Noh[1], Ros Gloag[2] and Naomi E. Langmore[1]

[1]Research School of Biology, Australian National University, RN Robertson Building, 46 Sullivans Creek Road, Canberra, Australian Capital Territory 2601, Australia
[2]School of Life and Environmental Sciences, University of Sydney, Macleay Building A12, Science Road, Sydney, New South Wales 2006, Australia

H-JN, 0000-0003-4937-9093; RG, 0000-0002-2037-4267; NEL, 0000-0003-3368-6697

Brood parasitic cuckoos lay their eggs in other birds' nests, whereafter the young cuckoo hatches, ejects its nest-mates and monopolizes the care of the host parents. Theory predicts that hosts should not evolve to recognize and reject cuckoo chicks via imprinting because of the risk of mistakenly imprinting on a cuckoo chick in their first brood and thereafter always rejecting their own chicks. However, recent studies have revealed that some hosts do reject cuckoo chicks from the nest, indicating that these hosts' recognition systems either do not rely on first brood imprinting, or use cues that are independent of chick phenotype. Here, we investigate the proximate mechanisms of chick rejection behaviour in the large-billed gerygone (*Gerygone magnirostris*), a host of the little bronze-cuckoo (*Chalcites minutillus*). We find that gerygones use true template-based recognition based on at least one visual chick trait (the number of hatchling down-feathers), and that this is further mediated by experience of adult cuckoos at the nest during egg-laying. Given the theoretical constraints of acquiring recognition templates via imprinting, gerygones must possess a template of own-chick appearance that is largely innate. This true recognition has facilitated the evolution of very rapid hatchling rejection and, in turn, striking visual mimicry of host young by little bronze-cuckoo chicks.

1. Introduction

Brood parasitic cuckoos impose heavy costs on their hosts, selecting for the evolution of host defences against parasitism [1–3]. The most widespread defence is egg rejection, and many hosts have evolved highly refined abilities to detect and eject eggs that differ in appearance from their own [4,5]. Curiously, however, these same hosts typically fail to reject the parasitic chicks once hatched, despite the imposters having a clearly distinct phenotype from the host's own young [2,6]. Several theoretical solutions to this long-standing puzzle have been proposed (reviewed in [7]). One explanation is that the costs of recognition errors may constrain the evolution of learned cuckoo chick discrimination in hosts wherever cuckoos evict the host eggs from the nest soon after hatching [8]. Lotem suggested that if hosts learn the appearance of their own chicks through imprinting on their first brood, a host parasitized during its first breeding attempt would falsely imprint on the lone foreign chick as its own young and thereafter reject its own offspring for the rest of its life [8]. The same problem would not impede the evolution of egg rejection, because even parasitized hosts are exposed to some of their own eggs during the egg-laying and incubation phases.

Lotem's hypothesis provides an explanation for the lack of true learned recognition of cuckoo chicks (assessment of the match between the template for a hosts' own young and the phenotype of the parasite chick) by hosts [8]. However, some hosts have evolved the ability to discriminate cuckoo chicks using 'recognition-free' mechanisms [9–11]. Recognition-free discrimination involves identifying the parasite chick from cues other than chick phenotype,

thereby avoiding the risk of mis-imprinting [7,10,12]. It has been shown to be the primary process operating in two hosts of evicting cuckoos. Hosts of Horsfield's bronze-cuckoo (*Chalcites basalis*) use the presence of a lone chick in the nest and the presence of adult cuckoos in the population as cues for abandoning parasitized nests [9]. Similarly, reed warbler (*Acrocephalus scirpaceus*) hosts of common cuckoos (*Cuculus canorus*) cue into the duration of parental care, abandoning chicks that remain in the nest for longer than the typical host nestling period [10]. These studies demonstrate that recognition-free discrimination provides hosts with a pathway for cuckoo chick rejection that circumvents the costs of mis-imprinting. Our aim is to test whether cuckoo chick discrimination can also evolve through true recognition, despite the theoretical costs of mis-imprinting proposed by Lotem [8]. One plausible way in which this could occur is if discrimination is largely innate, rather than learned [11]. In theory, true recognition has a significant advantage over some recognition-free mechanisms, because it can take place immediately upon hatching of the parasite chick, allowing the host to remove the cuckoo before it evicts host young. To date, no studies have demonstrated true recognition of parasite young. However, indirect evidence for this mechanism in some hosts stems from the apparent nestling or fledgling mimicry of host young by cuckoos [13–16]. Just as occurs at the egg stage, selection for mimicry might arise through host rejection of chicks with non-matching phenotypes [7,15].

The gerygone (*Gerygone* spp.) hosts of Australia's little bronze-cuckoos (*Chalcites minutillus*) are strong candidates for using true recognition of chicks. Despite typically suffering high parasitism rates, gerygones do not reject bronze-cuckoo eggs [13,14,17]. Instead, gerygones have the most effective known form of chick rejection because they reject cuckoo chicks by dragging them out of the nest within hours of hatching [13,14,18], sometimes succeeding in removing the cuckoo nestling (a nest-mate evictor) before it has a chance to evict the host young from the nest. Most Australian bronze-cuckoo species lay non-mimetic eggs, but their chicks are excellent visual mimics of host young, with each subspecies matching the colour of nestling skin, rictal flange and down-feathers of their favoured host species [15,19,20]. The little bronze-cuckoo is a particularly accurate mimic of host young [15], and it is unique among cuckoos in displaying multi-barbed nestling down-feathers, which are typical of passerine nestlings including their hosts, but are otherwise unknown in the cuckoo family [15].

Here, we use experimental manipulations to establish for the first time the mechanisms by which gerygones recognize and reject little bronze-cuckoo nestlings. We test for three non-mutually exclusive recognition-free cues (hatch order, the presence of an adult cuckoo and discordancy) and one true recognition cue (nestling down-feathers) that may be used to facilitate chick rejection. The presence of an adult cuckoo in the nest's vicinity has been shown to be an important component of chick rejection decisions in another bronze-cuckoo host [11]. Hatch order is also a possible recognition-free cue [21], because cuckoo eggs typically require a shorter incubation period and usually hatch 1–2 days before gerygone young [22]. However, this cue would only be useful in conjunction with another cue, indicating that the nest has been parasitized. Recognition by discordancy involves assessment of the differences between chick phenotypes within the same brood and rejection of the least common phenotype [23,24]. In the absence of true recognition, discordancy might favour visual mimicry of host young by cuckoos, provided that the cuckoo and host chicks are present in the nest together prior to rejection, and that host chicks typically outnumber cuckoos.

2. Material and methods

(a) Study area and species

We carried out our study from August to December 2016 along creeklines in and around Cairns, Queensland, Australia (16°55′ S, 145°46′ E) on a population of large-billed gerygones (*Gerygone magnirostris*) that experience high rates of parasitism by little bronze-cuckoos (63–65% [17]; this study). Little bronze-cuckoos were seen or heard, and parasitism occurred, at all creeks in the study. The large-billed gerygone builds untidy domed nests using grass, moss and spiders' egg-sacks, usually overhanging water [25]. Gerygones lay one egg every second day over a period of 4–8 days (average clutch: mean ± s.e. = 3 ± 0.09, range: 1–5, $n = 100$) and start incubation when their clutch is complete [25]. Cuckoos lay a single egg per host nest, during or shortly after the hosts' egg-laying period, and usually remove one host egg during the same visit. Two or three different females may lay in the same host nest [17,26]. Hosts mob the cuckoo if it is detected during laying, but mobbing has not been observed to prevent parasitism [17].

(b) General experimental methods

We located 54 large-billed gerygone nests during the nest-building phase by searching along creeks, rivers and lakes. Of these nests, 35 (65%) were subsequently parasitized by one ($n = 30$) or two ($n = 5$) cuckoos, and 19 were not parasitized. We checked nest contents daily to allow clutch manipulation as soon as eggs appeared and before incubation began. From hatching day, we monitored all 54 nests to determine whether nestling rejection occurred. Parasitized nests were filmed or observed continuously from hatching until host chick eviction (by cuckoo chicks) or cuckoo chick rejection (by host parents) occurred. When cuckoo chicks evicted host young and became the sole occupant of the nest, we continuously monitored the nest for at least a further 2 days during daylight hours to document any chick rejection or nest predation. In total, 19 nests (16 parasitized and three unparasitized) were monitored from 06.00–07.00 to 17.00–18.00 by an observer in a hide (approx. 5 m from nest) using binoculars, 17 nests (16 parasitized and one unparasitized) were filmed continuously with a video camera (Panasonic, HC-VX870M) and the remaining 20 nests (three parasitized and 15 unparasitized) were monitored with daily nest checks to determine whether any chicks were missing. If a host chick was missing from an unparasitized nest, we concluded that the host had rejected the chick. This conclusion is based on the lack of observations of partial predation in our study site (other than when an egg was stuck to or embedded in the lining of a depredated nest) and the fact that nest predation usually results in nest damage as the predator forces entry into the dome nest. If a nest check revealed that all chicks in the nest were missing, we concluded that the nest had been predated. We excluded three unobserved disappearances of cuckoo chicks from our analyses, because in each case the cuckoo chick was alone in the nest so we could not determine whether it disappeared due to ejection or predation. Three host chicks that died in the nest on the day after hatching day were excluded from the analysis because we have no evidence either for or against the idea that dead chicks were rejected. We calculated 'time to ejection' to the nearest day (hatch day = 0 days). In our experiments, we first

manipulated exposure of hosts to an adult cuckoo using a cross-fostering experiment, and then randomly assigned nests to (i) a hatch-order manipulation experiment, (ii) a feather trimming experiment, or (iii) both the hatch order and the feather trimming experiment (see electronic supplementary material, figure S1).

(c) Manipulation of opportunity to observe an adult cuckoo at the nest

To assess whether hosts' exposure to an adult cuckoo at the nest influenced chick rejection rates, we cross-fostered cuckoo eggs from some parasitized nests to unparasitized nests to create two conditions among nests containing one or two cuckoo eggs: naturally parasitized, such that parents had the opportunity to observe a cuckoo lay at their nest (nests: $n = 22$, chicks: $n = 25$), and artificially parasitized nests where adults did not see a cuckoo lay in their nest (nests: $n = 13$, chicks: $n = 15$). Two sources of evidence suggest that parents of naturally parasitized nests are likely to have had the opportunity to observe a cuckoo entering the nest. First, we filmed parasitism of the nest on three occasions and, in every case, the gerygone parents mobbed the cuckoo [17]. Second, although we cannot be certain that all naturally parasitized hosts observed the cuckoo during parasitism, it is certain that more gerygones in the 'naturally parasitized' group will have seen or interacted with a cuckoo at their nest than did gerygones in the 'artificially parasitized' group.

(d) Manipulation of hatch order and discordancy

Cuckoo eggs usually hatch 1–2 days before host eggs [22]. To determine the effect of hatch order on chick rejection, we delayed the hatching of cuckoo eggs ($n = 12$) by 5 days. We removed each freshly laid cuckoo egg and stored it in a cool, dark place. We replaced it temporarily with a non-viable gerygone egg, which had been collected from a depredated or abandoned nest (depredated nests sometimes contained intact eggs, if they were stuck to the nest lining). After 2 days of incubation, we removed the dummy egg and returned the cuckoo egg to the nest, such that any host eggs in the nest hatched 1–2 days before the cuckoo chick. As a control, we used the same procedure to remove and later replace a single gerygone egg from unparasitized nests ($n = 14$). Five cuckoo chicks also hatched later than host young naturally and these were included in the dataset.

When a cuckoo chick or chicks are the minority species in the brood, hosts may discriminate via discordancy and reject the most dissimilar chick or chicks. This recognition-free mechanism requires that cuckoo and host chicks are present in the nest at the same time, and that host chicks reliably outnumber cuckoos. In combination, our cross-fostering and hatch-order manipulations varied the composition of chicks in the nest at the same time, and thus allowed us to test for evidence of discordancy as a rejection cue by comparing rejection rates when the cuckoo chick was ($n = 8$) or was not ($n = 32$) the brood's minority species.

(e) Manipulation of chick morphology

To assess whether gerygones' rejection of cuckoo chicks is based on true recognition, and specifically on the recognition of nestling down-feathers, we manipulated hatchlings' feathers in a subset of nests ($n = 32$). On the day of hatching, we used nail scissors to trim the down-feathers of either one cuckoo chick ($n = 13$, including one naturally naked cuckoo chick) or one gerygone chick (unparasitized nests: $n = 16$, parasitized nests: $n = 4$) in the nest (figure 1). We compared the rate of rejection of trimmed chicks with that of chicks that were handled on hatching day, but did not have their feathers trimmed. We also counted the number of down-feathers of all chicks on hatch day, prior to the manipulation, to quantify natural variation in feather density and weighed chicks four times (hatching day, and 3, 7, and 13 days old) to test whether the manipulation otherwise affected chick growth.

(f) Statistical analyses

We used a generalized linear model (GLM) with a binomial distribution and a logit link using all chicks (full dataset) to assess parental responses to the chicks (accept/reject) according to (i) the presence or absence of down-feathers, (ii) hatching order, (iii) whether or not host was exposed to adult cuckoo and/or (iv) whether or not a cuckoo chick was in the minority in the nest. The independent variables were species (cuckoo or host), the four manipulations (all scored as yes/no: hatched first, feathers trimmed, naturally parasitized cuckoo visited the nest and cuckoo chick in the minority), hatching date and the two-way interactions between these variables. We also tested the quadratic term for the hatching date because seasonal trends are often nonlinear, but the result was the same. Initially, we attempted to run a mixed model controlling for nest identity as a random effect because there were multiple chicks in each nest, but this made the model unstable due to the small number of replicates in each nest. Instead, we ran a binomial GLM with a logit link function on a reduced dataset comprising only one experimental chick per nest (reduced dataset) and then compared the results from the full dataset and the reduced dataset. Where there was only one manipulated chick in the nest, this was included in the reduced dataset. If there were two experimental chicks or there was no manipulated chick in the nests, we selected one chick randomly. In addition, to identify which of these factors contributed significantly to the time to rejection, we used a GLM with a binomial distribution depending on whether or not they were rejected on the day of hatching using all rejected chicks. The independent variables were the same as in the former GLM analysis. Owing to the controversy over whether null hypothesis testing or information theoretic approaches are better for analysis of experimental studies [27], we used both methods. We applied a backward-elimination procedure (tables 1 and 2), and the Akaike information criterion (AIC) was also used to support selection of the final model (best-fit model) (electronic supplementary material, tables S1 and S2). The results did not differ depending on the approach used; the significant effects as identified by the backward-elimination procedure were the same as the best model using AIC. We also evaluated multicollinearity using the variance inflation factor (VIF) in the models, and all VIF values were lower than the suggested threshold (greater than 10 [28]). All statistical analyses were performed using R software v. 3.4.3 [29].

3. Results

Our full dataset (all chicks in experimental nests) included 85 host chicks and 40 cuckoo chicks across 54 nests. During the course of our experiment, 36 chicks (both host and cuckoo) were rejected from 32 nests (although host chicks were only rejected following down-feather manipulations; see below). We captured nine rejection events on film at eight nests (see example in electronic supplementary material, video S1), and a further five rejection events were observed with binoculars. The remaining chick rejections by hosts were inferred from daily nest checks. In all filmed or observed cases, large-billed gerygones pulled living chicks out of the nests, and the parents then continued to care for the remaining eggs and nestlings. Ejected chicks were either dropped just under the nests or carried up to 3 m from the nest before being dropped.

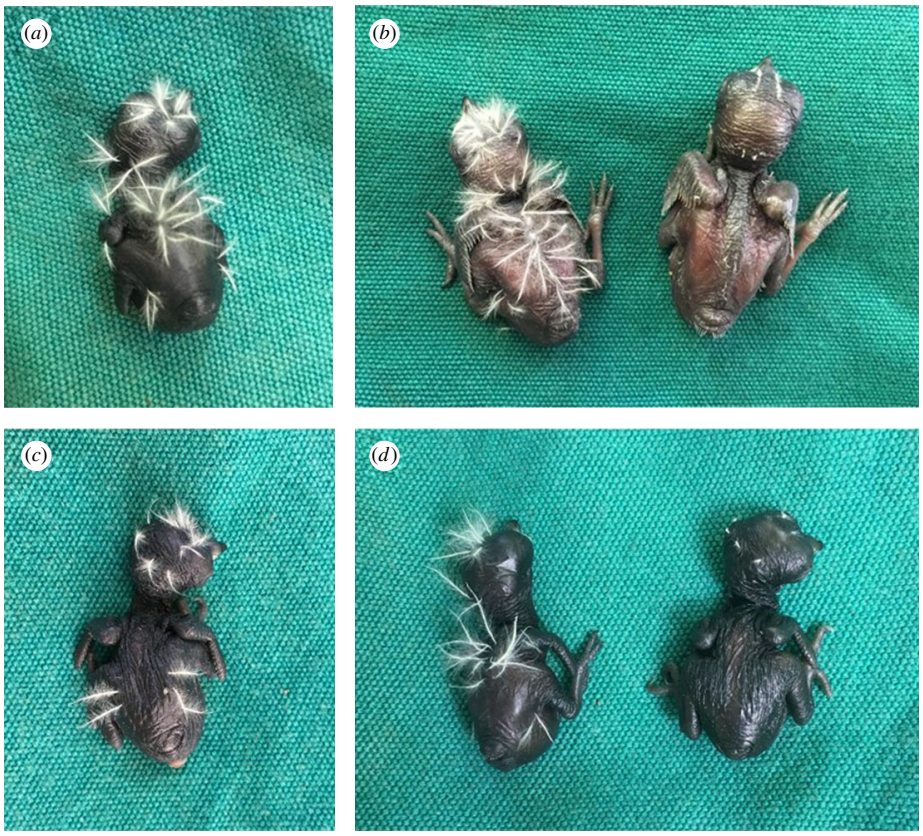

Figure 1. Photographs of large-billed gerygone and little bronze-cuckoo chicks. (*a*) An untrimmed gerygone on hatching day. (*b*) An experimental brood comprising one untrimmed host chick (left) and one trimmed host chick (right, both 3 days old). (*c*) An untrimmed cuckoo on hatching day. (*d*) An experimental brood comprising one untrimmed host chick (left) and one trimmed cuckoo chick (right, both on hatching day).

(a) True recognition

Chick species and the presence of nestling down-feathers were significant predictors of their rejection by gerygone hosts; cuckoos were more likely to be rejected than host chicks, trimmed chicks more likely to be rejected than untrimmed chicks in cuckoos and untrimmed host chicks were never rejected (table 1 and figure 2*a*). Hosts removed 93% of trimmed cuckoo chicks (13 of 14 chicks) and 56% of trimmed host chicks (11 of 20 chicks; figure 2*a*). Among untrimmed chicks, 50% of cuckoo chicks were rejected (14 of 28), while untrimmed host chicks were never removed ($n = 65$ chicks, figure 2*a*), and trimming was the only manipulation that resulted in hosts rejecting their own chicks. Where cuckoo chicks did not have their down-feathers manipulated, host parents showed a non-significant tendency to reject those that had naturally fewer down-feathers (rejected: mean $\pm$ s.e. $= 13 \pm 1.77$, $n = 14$; accepted: mean $\pm$ s.e. $= 17.07 \pm 1.67$, $n = 14$; Student's *t*-test: $t = -1.67$, $p = 0.1068$). Similarly, our results from the reduced dataset showed that chick species and the presence of down-feathers were the most significant predictors of rejection (table 1).

The timing of nestling removal further supports a role for direct species-specific chick cues in gerygones' rejection decisions. Cuckoo chicks were more likely to be rejected on the hatch day than host chicks, but whether or not a chick was trimmed did not influence the timing of its removal (table 2). All rejected cuckoo chicks were removed by hosts within 2 days of hatching, with 56% (14 of 25) and 36% (9 of 25) rejected on hatching day and the next day, respectively. Only 8% (2 of 25) were rejected 2 days after hatching (electronic supplementary material, figure S2). By contrast, just 18% (2 of 11) of rejected host chicks were ejected on hatch day, 36% (4 of 11) were removed the day after hatching and 46% (5 of 11) were rejected 2–3 days after hatching (electronic supplementary material, figure S2). In the case of cuckoo chicks, rapid rejection was necessary to preserve the host young in the nest: when hosts removed cuckoo chicks on hatch day, none of their own nestlings had yet been evicted by the cuckoo chick, while those cuckoo chicks rejected on later days had already removed some or all gerygone young (six out of 11).

Finally, we confirmed that hatchling down-feathers vary under natural conditions in both gerygone and cuckoo young. On average, large-billed gerygone nestlings had more down-feathers on the day of hatching (mean $\pm$ s.e. $= 37.35 \pm 1.45$, range: 23–68, $n = 41$, one chick per nest) than little bronze-cuckoo nestlings (mean $\pm$ s.e. $= 14.40 \pm 1.19$, range: 0–29, $n = 38$, Student's *t*-test: $t = -12.226$, $p < 0.0001$). The variation in the number of down-feathers of gerygone chicks within the same brood was significantly less than that between broods (one-way ANOVA: $F_{40,44} = 5.35$, $p < 0.0001$).

(b) Recognition-free cues in host decisions to reject chicks

In naturally parasitized nests in which hatch order was not manipulated ($n = 22$), the cuckoo hatched before the host

Table 1. Effects of experimental treatment on chick rejection. Acceptance or rejection of nestlings was modelled as a binomial in a GLM with a logit link function on both the full dataset and the dataset including only one manipulated chick in each nest (results in parentheses). Significant *p*-values are shown in italic.

				95% CI			
term	effect	estimate	s.e.	LCI	UCI	deviance	*p*-values
included	species (cuckoo)	−1.578 (−0.264)	0.777 (0.506)	−3.454 (−1.122)	−0.239 (0.565)	31.667 (5.969)	*<0.0001* (*0.015*)
	species (host)	−6.020 (−3.170)	1.370 (1.080)	−9.520 (−5.498)	−3.861 (−1.702)		
	trim (y)	6.252 (3.174)	1.441 (1.084)	3.893 (1.675)	9.840 (5.500)	41.478 (16.372)	*< 0.0001* (*< 0.0001*)
	exposure to adult cuckoo (y)	2.269 (1.273)	0.924 (0.939)	0.601 (−0.187)	4.355 (2.994)	2.108 (2.037)	0.146 (0.154)
	trim (y): exposure to adult cuckoo (y)	−4.109 (−3.069)	1.62 (2.267)	−7.798 (−7.131)	−1.1690 (0.581)	3.920 (−1.924)	*0.005* (0.165)
excluded	hatch order (first)	−0.0144 (−0.828)	0.6391 (0.794)	−1.094 (−2.197)	1.027 (0.444)	−0.001 (−1.130)	0.982 (0.288)
	discordancy (y)	0.388 (0.837)	0.764 (0.844)	−0.868 (−0.526)	1.673 (2.287)	−0.259 (−1.011)	0.6111(0.315)
	hatching date	0.013 (0.011)	0.012 (0.014)	−0.006 (−0.013)	0.035 (0.037)	−1.287 (−0.572)	0.256 (0.450)
	species (h) : hatch order (f)	−2.864 (3.100)	3831.454 (1.780)	−80.844 (0.343)	75.116 (6.331)	−0.000 (−3.458)	1 (0.063)
	species (h) : trim (y)	17.168 (34.63)	2045.330 (9300)	−77.750 (−)	515.633 (−)	−1.859 (−0.826)	0.173 (0.364)
	species (h) : exposure to adult cuckoo (y)	−2.332 (16.335)	3488.828 (2655.399)	−67.924 (−)	59.085 (−)	0.000 (−0.676)	1 (0.411)
	species (h): discordancy (y)	−17.307 (−18.476)	2327.470 (2711.847)	−634.712 (−)	84.847 (−)	−1.693 (−2.094)	0.193 (0.150)
	trim (y) : hatch order (y)	−1.510 (−19.058)	1.702 (4091.637)	−4.614 (−)	1.166 (−)	−0.839 (−1.690)	0.360 (0.194)
	trim (y) : discordancy (y)	1.659 (−)	17970 (−)	−327.640 (−)	344.594 (−)	−0.000 (−)	1 (−)
	hatch order (y) : exposure to adult cuckoo (y)	−1.326 (14.62)	1.370 (15490)	−3.679 (403.428)	0.886 (360.749)	−0.963 (0.000)	0.326 (1)
	hatch order (y) : discordancy (y)	3.115 (0.967)	1.991 (1.822)	−0.026 (−2.071)	6.700 (4.015)	−2.659 (−0.281)	0.103 (0.596)
	exposure to adult cuckoo (y) : discordancy (y)	−1.258 (11.97)	1.918 (30940)	−4.531 (−804.412)	2.061 (780.835)	−0.422 (−0.000)	0.516 (1)

Table 2. Effects of experimental treatment on time until rejection for those nests in which hosts rejected a chick. Time to rejection was modelled as a binomial (hatching day = 0, one or more days post-hatching day = 1) in a GLM with a logit link function and the dataset includes all the rejected cuckoo and host chicks. Significant *p*-values are shown in italic.

			95% CI			
term	effect	estimate (s.e.)	LCI	UCI	deviance	*p*-values
included	species (c)	0.241 (0.403)	−0.418	0.919	45.780	*0.008*
	species (h)	−1.792 (0.764)	−3.292	−0.682		
excluded	trim (y)	−0.942 (0.920)	−2.547	0.528	−1.095	0.295
	hatch order (f)	0.542 (0.900)	0.282	0.890	−0.368	0.544
	exposure to adult cuckoo (y)	−1.138 (0.981)	−2.961	0.366	−1.508	0.219
	discordancy (y)	−1.212 (1.211)	−3.652	0.629	−1.136	0.287
	hatching date	0.010 (0.017)	−0.017	0.039	−0.368	0.544
	species (h) : trim (y)	1.005 (4696)	—	—	−0.000	1
	species (h) : hatch order (y)	1.854	—	—	−2.866	0.091
	species (h) : exposure to adult cuckoo (y)	54.95 (21670)	—	—	0.000	1
	species (h) : discordancy (y)	20.54 (4212)	—	—	−2.885	0.089
	trim (y) : hatch order (y)	18.820 (5628)	—	—	−1.253	0.263
	trim (y) : exposure to adult cuckoo (y)	15.848 (4027.416)	—	—	−0.272	0.602
	hatch order (f) : exposure to adult cuckoo (y)	16.920 (3810.961)	—	—	−0.624	0.429
	hatch order (f) : discordancy (y)	2.117 (24670)	—	—	-1.805×10^{-9}	1
	exposure to adult cuckoo (y) : discordancy (y)	55.100 (32090)	—	—	-1.723×10^{-8}	1

chicks in 77% of cases, but based on experimental nests, hatch order had no significant effect on the probability of chick rejection (table 1). Whether the cuckoo chick was in the minority in the brood also did not influence chick rejection decisions (table 1). In addition, hatch order and whether or not a chick is in the minority in the brood did not affect the timing of its removal (table 2).

Host's rejection decisions were influenced in part, however, by the exposure to adult cuckoos in interaction with chick phenotype (table 1), with hosts more likely to reject a cuckoo chick if it had been laid naturally into the nest than if it had been cross-fostered there from another nest by us (table 1 and figure 2*b*). This was clearly evident among the sample of untrimmed cuckoo chicks; only 18% of untrimmed cuckoo chicks (two out of 11) from artificially parasitized nests were ejected, whereas parents that had the opportunity to observe adult cuckoos laying rejected 69% of untrimmed cuckoo chicks (11 out of 16; Fisher's exact probability test: $p < 0.01$; figure 2*b*). However, our results from the reduced dataset showed that the effect of whether the nest was parasitized naturally or artificially was trivial (table 1), presumably due to dataset sample size differences.

4. Discussion

Hosts that reject parasite nestlings may do so based either directly on chick phenotype (true recognition) or on recognition-free cues. True recognition is assumed to be maladaptive for cuckoo hosts if it relies on an imprinted template [8], and previous studies have found experimental support only for recognition-free mechanisms [9,10]. Our results, however, provide the first experimental evidence that hosts can use true recognition when rejecting foreign nestlings, as large-billed gerygones regularly rejected nestlings that differed from their own offsprings' phenotype due to a lack of hatchling down-feathers. Gerygones combined this use of phenotypic cues with at least one additional chick-recognition-free cue, being more likely to reject cuckoo chicks when they had the opportunity to witness an adult cuckoo laying in the nest.

(a) Chick rejection based on true recognition

At least to the human observer, the number of down-feathers present on newly hatched chicks is the most obvious morphological cue available for discriminating between own and parasitic young; most host chicks have significantly more down-feathers than cuckoo chicks. Gerygones too were confirmed to use this cue in rejection decisions, being prompted to reject cuckoos, and even some of their own young, for which down-feathers were artificially removed. However, trimmed cuckoos were rejected at far higher rates than trimmed host young, indicating that gerygones use additional, as yet unidentified phenotypic cues. The begging calls of newly hatched chicks were audible to the human ear (H.-J.N. 2016, personal observation) and parents frequently made provisioning visits to the nest before they removed the chicks, so differences in begging call structure are a possible cue that warrants further investigation.

True recognition requires that hosts possess an internal template of the acceptable chick phenotype, to which they are able to compare cuckoo chicks. Given that to acquire

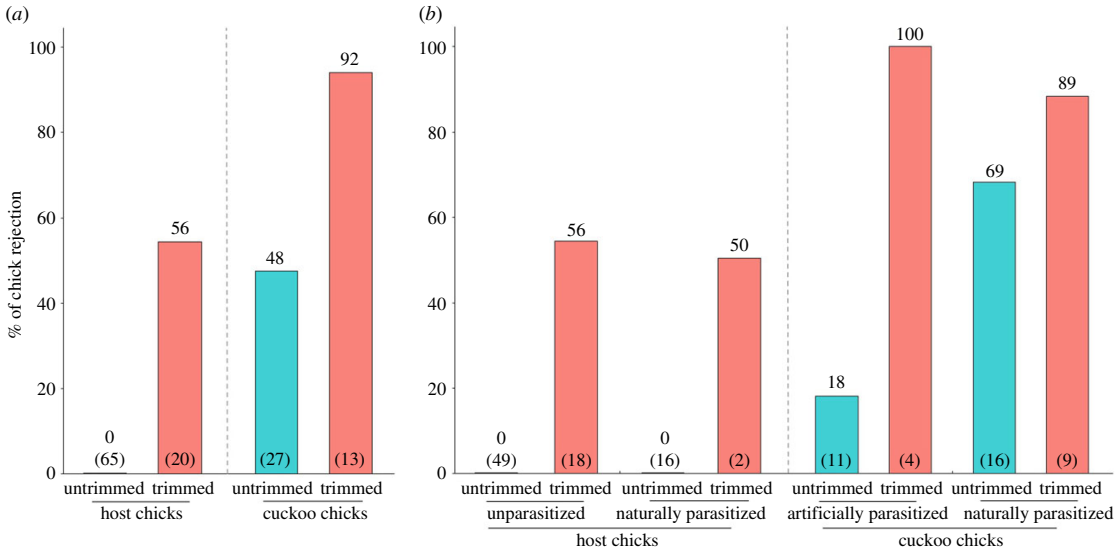

Figure 2. (*a*) The percentage of large-billed gerygone and little bronze-cuckoo chicks that were ejected according to whether down-feathers were trimmed. (*b*) The percentage of untrimmed and trimmed chicks that were ejected among host and cuckoo nestlings according to whether the nest was naturally or artificially parasitized (i.e. whether an adult cuckoo visited the nest during the egg-laying period). Sample sizes are given in parentheses at the base of the bar, and numbers above bars depict the exact percentage.

this template solely through experience with a first brood would lead to maladaptively high rates of recognition error in the host of an evicting cuckoo [8], a gerygone's template must have an alternative origin. One possibility is that chick templates are largely innate, driven by strong selection for correct identification of own and parasitic young. Such innate templates could still be refined through experience, in much the same way as songbirds have an innate template for their species's song that is refined through interactions with conspecifics [11]. Rejection decisions can then be further refined through the complementary use of recognition-free cues (discussed below). The resulting recognition and rejection system is certainly effective for large-billed gerygones in our study area, as we never observed the mistaken rejection of host young (other than those that were trimmed). Notably, however, Sato *et al*. [13] reported several cases of large-billed gerygones rejecting their own nestlings in a different study population, so it remains unclear whether low error rates are a general feature of gerygones' chick rejection behaviour. Recognition errors are most likely to occur in situations in which mimicry is highly accurate. In our study population, mimicry by little bronze-cuckoos was imperfect, because they had fewer nestling down-feathers than large-billed gerygone nestlings. However, host rejection was influenced more by the presence/absence of down-feathers than by the number of down-feathers *per se*. In addition, we found lower variation within than between broods in host down-feather abundance. This suggests that gerygones may be under selection for low intra-brood variation in the number of down-feathers to facilitate detection of cuckoo chicks, in much the same way as some other cuckoo hosts may experience selection for low intra-clutch variation in egg phenotype, facilitating detection of cuckoo eggs [30–33]. Such a process would require either that host and cuckoo chicks were present in the nest together (which occurs in a minority of nests) or that hosts remember their own chick morphology from previous broods.

Some combination of innate true recognition and more flexible mechanisms in gerygones' chick rejection would be consistent with our understanding of egg rejection mechanisms. Egg rejecter species show variation within and between populations in the form and extent of egg rejection behaviour [24,34], and individual hosts' reactions towards foreign eggs may also vary with conditions or experience [35]. The existence of both consistent and flexible patterns of egg rejection behaviour implies that both innate and learning mechanisms can be involved [34], and many host species appear to combine one or more variants of the true recognition process with proximate context-dependent factors when making rejection decisions [24]. Both egg rejection and chick rejection thus seem to be complex processes, using considerable mechanistic variation within and between species.

(b) Recognition-free discrimination

Gerygones were more than twice as likely to reject a cuckoo chick if an adult cuckoo had visited the nest during the egg-laying period than if the nest was parasitized artificially, indicating that the opportunity to observe or interact with a cuckoo at the nest strongly influenced rejection behaviour, as has also been observed in another bronze-cuckoo host [11]. Moreover, our results showed the strongest effect of exposure to adult cuckoos in interaction with chick phenotype (table 1), suggesting that this cue on its own is not enough to prompt rejection and must be coupled with cues from the chicks themselves. This indicates that the combination of this contextual cue with one or more phenotypic cues may allow gerygones to substantially reduce the risk of mistakenly rejecting their own young, particularly given the accurate host–cuckoo nestling mimicry in this system [36–39].

Notably, if hosts use the presence of adult cuckoos as a cue to reject nestling cuckoos, the cue is 'recognition-free'

with respect to chick phenotype, but does require the recognition of adult cuckoos. Based on behavioural responses, large-billed gerygones readily distinguish between adult cuckoos near their nests, which elicit mobbing, and predators or harmless species, which do not (F. Jacomb *et al.* 2015, unpublished data) [40]. Although it is unknown whether mobbing ever succeeds in preventing a cuckoo from laying, our results indicate that the recognition of adult cuckoos has an important role in gerygones' antiparasite defence, by increasing the accuracy of chick rejection decisions. Accordingly, our study provides support for strategy facilitation [41], in which adaptations at one stage of the evolutionary arms race (in this case, the egg-laying stage) promote the evolution of defences at another stage (the nestling stage).

We found no evidence that large-billed gerygones use two other candidate recognition-free cues: hatch order or discordancy. A simple 'reject the odd one out' rule is useful only when there are multiple chicks in the nest, and only one of these is a cuckoo, a condition that is rarely met in large-billed gerygones owing to the shorter incubation period of cuckoo nestlings and the small clutch size of gerygones. A strategy of 'reject the first hatched chick' would, in theory, be relatively effective for gerygones in ridding themselves of cuckoos, particularly if enacted only when adult cuckoos have been seen at the nest. However, the occurrence of multiple parasitism in this system (approx. 30% of all parasitized nests receive multiple cuckoos eggs [17,42]) reduces the benefit of such a rule of thumb substantially, because often another cuckoo will simply hatch to take the place of the rejected one.

(c) Implications for cuckoo–host coevolution and diversification in little bronze-cuckoos

Our results provide the first experimental demonstration that host defences can select for the evolution of nestling mimicry in a brood parasite. Previous work revealed that the nestlings of three bronze-cuckoo species are near perfect visual mimics of the host chicks they exploit [15]. Moreover, one host, the superb fairy-wren *Malurus cyaneus*, was less likely to reject cuckoo chicks of a species that specializes on fairy-wrens (Horsfield's bronze-cuckoo *C. basalis*) than a cuckoo species that uses fairy-wrens rarely (the shining bronze-cuckoo *Chalcites lucidus*) [9]. However, only recognition-free cues for chick discrimination were identified in this system, so it was unclear whether host rejection selected for mimicry of host young [11]. Furthermore, some forms of chick mimicry might arise for reasons other than host rejection [43], such as to exploit biases in host–parent communication and extract the optimal resources from host parents [44]. While it remains possible that the visual mimicry of little bronze-cuckoos also increases host provisioning rates, it seems likely that it has been primarily driven by gerygones' chick rejection behaviour.

In this study, our focus was demonstrating that true nestling recognition can evolve, contrary to the predictions of theory based on an imprinting model of chick rejection [8]. Mis-imprinting constraints are not the only explanation, however, for the apparent scarcity of chick rejection across hosts of brood parasites. Effective rejection of cuckoo eggs can prevent the evolution of cuckoo chick rejection by making the cuckoo nestling a 'rare enemy', such that the benefits of discriminating against it are outweighed by the costs of recognition errors [7,45,46]. Curiously, large-billed gerygones do not reject foreign eggs even though little bronze-cuckoo eggs look very different from their own. This is surprising given that hosts suffer fewer costs of parasitism by implementing defences early in the breeding cycle rather than later. Indeed, three non-mutually exclusive explanations for this are that (i) egg rejection is constrained by poor visibility inside the nest, because dark-coloured bronze-cuckoo eggs are cryptic inside dark host nests [17,26], (ii) egg rejection is constrained by bill morphology, because cuckoo eggs are too large or thick-shelled to be ejected and methods of egg rejection that remove or abandon whole clutches are too costly [47–49], and (iii) hosts may benefit by delaying rejection of the parasite until the chick stage when there is a risk of multiple parasitism, because allowing the cuckoo egg to remain in the nest reduces the probability that a host egg will be removed during subsequent parasitism events (the egg dilution hypothesis [17,50]).

Different subspecies of the little bronze-cuckoo exploit different hosts, and cuckoo mimicry of host nestlings can extend even down to the level of subspecies [51]. For example, *C. m. minutillus* mimics the dark skin and white down of nestling large-billed gerygones [15], whereas *C. m. barnardi* mimics the pink skin and yellow down of the offspring of white-throated gerygones *Gerygone albogularis* [19,43]. In addition, the little bronze-cuckoo occupies a wider distribution and has more subspecies than any other *Chalcites* cuckoos (10 described subspecies, compared to just one to four variants of other bronze-cuckoos) [52]. Although the rejection behaviour of other little bronze-cuckoo hosts remains to be studied, it is plausible that the observed variation in little bronze-cuckoo chicks has evolved in response to true recognition and chick rejection by their hosts, ultimately reinforcing reproductive isolation among cuckoo populations that exploit different host species [53]. Thus, unlike recognition-free mechanisms of chick rejection, true recognition of cuckoo chicks may have significant consequences for the coevolutionary trajectory of their parasites, by driving host-specific genetic diversification in parasite populations.

Ethics. The aim of this study was to explore the mechanism of chick rejection. While chick rejection by hosts leads to the death of the rejected chick, this experiment did not increase the frequency of chick death, because in a parasitized nest either the cuckoo chick or the host chicks always die under natural conditions. Our experimental manipulations may have influenced whether it was the cuckoo or the host chicks that died, but overall the experiments did not cause mortality in more nests than would happen naturally. All experiments were conducted under approval of the Australian National University Animal Experimentation Ethics Committee Protocol number A2016/16.

Data accessibility. All data supporting this article are available from Dryad (http://dx.doi.org/10.5061/dryad.vv76g) [54].

Authors' contribution. H.-J.N. performed experiments and analyses and drafted the manuscript. H.-J.N., R.G. and N.E.L. conceived the study, discussed results, analyses and implications, and revised the manuscript.

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